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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	SAH et al	Confirmation No.:	8421
Serial No.:	09/134,771	Art Unit:	1636
Filed:	August 12, 1998	Examiner:	S. Kaushal
For:	HUMAN MESENCEPHALON CELL LINES AND METHODS OF USE THEREFOR	Attorney Docket No:	10624-009-999

BRIEF ON APPEAL

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Sir:

Pursuant to the provisions of 37 C.F.R. § 1.191 and § 1.192, an appeal is taken herein from the final rejection, dated September 20, 2002, which rejects claims 1-15 and 23-27 of this application. Appellants submit herewith: (a) an original and two copies of this Appeal Brief; and (b) a Petition for Extension of Time of one (1) month, from October 30, 2003 to November 30, 2003 with provision for the required fee.

REAL PARTY IN INTEREST

The real party in interest is the assignee of the above-identified application: Signal Pharmaceuticals, Inc. ("Signal") a company having a place of business at 4550 Towne Center Court, San Diego, California 92121. Signal is a wholly-owned subsidiary of Celgene Corp., a company having a place of business at 7 Powder Horn Drive, Warren, New Jersey 07059.

RELATED APPEALS AND INTERFERENCES

Appellants and their legal representatives hereby submit that they are not aware of any appeal or interference that directly affects, will be directly affected by, or will have a bearing on the Board's decision in this appeal.

STATUS OF THE CLAIMS

This application was filed with 24 claims. Claims 16-22 were canceled without prejudice pursuant to a Restriction Requirement (Paper No. 6), filed February 14, 2000). Claims 25 and 26 were added in an Amendment under 37 C.F.R. 1.116 (Paper No. 18), filed March 26, 2001. Claims 11 and 12 were canceled, and claim 27 added, in an Amendment under 37 C.F.R. 1.111 (Paper No. 24), filed June 19, 2002. Accordingly, claims 1-10, 13-15, and 23-27 of this application are under final rejection and are the subject of this appeal. The appealed claims are presented in Exhibit 1 attached hereto.

STATUS OF AMENDMENTS

Subsequent to the September 20, 2002 final Office Action, a Response under 37 C.F.R. § 1.116 was filed on March 20, 2003. In an Advisory Action, mailed April 14, 2003, the Examiner indicated that the Response was entered but did not place the application in condition for allowance for the reasons set forth in the Advisory Action.

SUMMARY OF THE INVENTION

The invention, as recited by the claims on appeal, satisfies the long-felt need in the art for human neural progenitor cell lines whose differentiation is regulatable. Before applicants' invention, researchers had identified human neural stem cells, but their culture and differentiation was difficult, and their use required the repeated use of fresh fetal material that is difficult to obtain. Methods of making immortalized rat neural stem cells were also known, but persons of skill in the art had no success in applying those methods to human neural stem cells. The claimed invention, as first practiced by the Inventors, encompasses a method of conditionally immortalizing human mesencephalon progenitor cells to produce immortalized cell lines, and of differentiating the conditionally-immortalized cells to produce mesencephalon cells. The invention further encompasses the conditionally-immortalized human mesencephalon cells themselves, and the mesencephalon cells differentiated therefrom. These cells have proven highly valuable to researchers interested in treating a wide spectrum of neurological disorders.

The invention as currently claimed is generally described on page 2, lines 5-30; page 4, line 29 to page 8, line 28; and in Example 1 and Figures 1-4. As claimed, the invention provides a method for producing a conditionally-immortalized human mesencephalon neural progenitor cell, comprising: (a) plating human mesencephalon cells on a first surface and in first growth medium that permits proliferation; (b) transfecting said progenitor

cells with DNA encoding a selectable marker and an externally regulatable growth-promoting protein; and (c) selecting an adherent monolayer of the transfected cells on a second surface and in a second serum-free growth medium that permits attachment and proliferation, wherein the second serum-free growth medium comprises FGF-2, EGF and PDGF, and therefrom producing a conditionally-immortalized human mesencephalon cells in which the growth-promoting protein is regulated by an external factor, such that suppression of the growth promoting protein results in differentiation of the cell into a neuron. (Page 2, lines 6-13; page 7, lines 23-25; page 16, lines 23-25.) The first and second surfaces may be independently selected from the group consisting of substrates comprising one or more of a polyamino acid, fibronectin, laminin or tissue culture plastic. (Page 2, lines 13-16; page 7, lines 19-22; page 8, lines 14-16.) The recited growth-promoting gene may be an oncogene (page 5, lines 24-27); the oncogene may specifically be v-*myc* (page 5, lines 24-25). In the method, expression of the growth-promoting gene may be inhibited specifically by tetracycline. (Page 6, lines 7-16; page 16, lines 14-20; page 17, lines 3-4, 10-11).

The invention also provides a conditionally-immortalized human mesencephalon neural progenitor cell capable of differentiation into neurons, wherein the cell is transfected with DNA encoding a growth-promoting protein that is regulated by an external factor, such that suppression of the growth-promoting protein results in differentiation of the cell into a neuron, and wherein the cell is polygonal and grows as an adherent monolayer. (Page 2, lines 19-22; page 7, line 23 to page 8, line 3; page 16, line 23 to page 17, line 2.) Such cells may be capable of differentiation into dopaminergic neurons (page 2, lines 19-21; page 8, lines 23-24; page 17, lines 20-26) or into GABA-ergic neurons (page 2, lines 21-22; page 7, lines 27-28; page 17, lines 26-27; Figure 4). The cell may be produced by the specific method described above. (Example 1.) Such a cell may be present within a clonal cell line. (Page 7, line 29 to page 8, line 3; 17, lines 7-24.)

The invention also provides a method for producing a neuron, comprising culturing a cell produced according to the method above in the presence of at least one differentiating agent under conditions that inhibit expression of the growth-promoting gene. (Page 2, lines 23-25; page 8, lines 4-12; page 17, lines 10-24.) In one aspect, the cell is cultured in medium comprising tetracycline. (Page 6, lines 7-16; page 16, lines 14-20; page 17, lines 3-4, 10-11.) The differentiating agent may comprise GDNF (page 2, lines 26-27; page 8, line 17-19; page 17, lines 11, 20-21); the combination of forskolin, GDNF and CNTF (page 2, lines 26-27; page 8, line 17-19); or the combination of forskolin, GDNF, CNTF, IGF-1 and BDNF (page 2, lines 26-27; page 8, line 17-21; page 17, lines 10-12, 20-21).

The invention further provides a neuron produced according to this method. (Page 2, lines 29-30; page 8, lines 23-28; page 17, lines 10-27.) The neurons may be dopaminergic (page 2, lines 19-21; page 8, lines 23-24; page 17, lines 20-26) or GABA-ergic (page 2, lines 21-22; page 7, lines 27-28; page 17, lines 26-27; Figure 4).

ISSUES ON APPEAL

(1) The first issue presented by this appeal is whether the novel method of conditionally immortalizing human mesencephalon stem cells, and differentiating these cells, is obvious under 35 U.S.C. § 103(a) over the combination of *five* references cited by the Examiner, *i.e.*, Hoshimaru *et al.*, *Proc. Natl. Acad. Sci. USA* 93:1518-1523 (1996) and Prasad *et al.*, *In Vitro Cell Devel.* 30A:596-603 (1994) in view of Boss *et al.*, U.S. Patent No. 5,411,883 (1995), Weiss *et al.*, U.S. Patent No. 5,750,376 (1998) and Gallyas *et al.*, *Neurochem. Res.* 22(5):569-575 (1997), even though (1) the combination does not teach all of the limitations of the claims; (2) the combination does not provide a reasonable expectation of success in practicing the claimed methods; and (3) there is no suggestion to combine the cited references.

(2) The second issue presented by this appeal is whether the novel conditionally immortalized human mesencephalon stem cells, and the mesencephalon cells differentiated therefrom, are obvious under 35 U.S.C. § 103(a) over the combination of *five* references cited by the Examiner (*see* (1), *above*) even though conditionally-immortalized human mesencephalon progenitor cells are substantially different biochemical entities from rat cells, and, as a result, there is no suggestion to combine the cited references.

GROUPING OF CLAIMS

Claims 1-5, 9, 10 and 25-27, directed to methods of producing conditionally-immortalized human mesencephalon progenitor cells and the neural cells differentiated therefrom, stand separately from claims 6-8 and 13-15, directed to the conditionally-immortalized mesencephalon progenitor cells and the neural cells differentiated therefrom.

REFERENCES RELIED UPON BY THE EXAMINER

Primary:

Hoshimaru *et al.*, *Proc. Natl. Acad. Sci. USA* 93:1518-1523 (1996) (submitted herewith as Exhibit 2) discloses the production of conditionally-immortalized rat neuronal progenitor cells by transfection with a retroviral vector having a tetracycline-controlled transactivator operably linked to a v-*myc* oncogene. When tetracycline is absent, the oncogene is active, and the rat progenitor cell containing it proliferates but does not differentiate. In the presence of tetracycline, however, the oncogene is inactivated, and the progenitor cell containing it may differentiate into a neural cell.

Prasad *et al.*, *In Vitro Cell Devel.* 30A:596-603 (1994) (submitted herewith as Exhibit 3) discloses immortalized clones of rat nerve cells derived from mesencephalon tissue. Cells from the rat mesencephalon were transfected with plasmids expressing the SV-40 large T antigen, a transforming protein, plated on a special substrate and in a special selection medium; surviving cells had characteristics of neurons.

Secondary:

Boss *et al.*, U.S. Patent No. 5,411,883 (1995) (submitted herewith as Exhibit 4) discloses a method of preparing neuron progenitor cells comprising obtaining mesencephalon tissue from an embryo, dissociation of the tissue to obtain single cells and cell clusters, culturing any progenitor cells present in a first culture medium that selects for the progenitor cells, and proliferating the progenitor cells in a second medium.

Weiss *et al.*, U.S. Patent No. 5,750,376 (1998) (submitted herewith as Exhibit 5) discloses a method for producing genetically modified neural cells. The reference discloses certain growth factors and growth conditions for culturing neural progenitor cells and neural cells.

Gallyas *et al.*, *Neurochem. Res.* 22(5):569-575 (1997) (submitted herewith as Exhibit 6) discloses the measurement of the concentrations of the neurotransmitters acetylcholine, γ-aminobutyric acid (GABA) and monoamines in immortalized rat or mouse cell lines.

ARGUMENT

The Examiner has failed to make out a *prima facie* case of obviousness against the pending claims. In particular, the Examiner rejects the claims over a combination of

several references that (1) does not provide a reasonable expectation of success; (2) does not teach all of the limitations of the claims; and (3) would not be combined by those of skill in the art because there is no suggestion to combine them. In arguing that the combination of references does so, the Examiner has, respectfully, misunderstood the teachings of several of the cited references. Moreover, the Examiner attempts to combine art relating to rat neuronal cells with art relating to human mesencephalic cells, a combination that attempts to combine two different cell types, from the different species, recognized in the art as being substantially different from each other.

At the outset, Applicants address the Examiner's contention that "[Applicants'] arguments taken as a whole rely heavily on the deficiencies of each reference taken alone. One cannot show non-obviousness by attacking references individually where the rejections are based on combinations of references," citing *In re Keller*, 642 F.2d 413, 426, 208 U.S.P.Q. 871, 882 (C.C.P.A 1981) and *In re Merck & Co, Inc.*, 800 F.2d 1091, 1097, 231 U.S.P.Q. 375, 380 (Fed. Cir. 1986). The Examiner misconstrues Applicants' arguments. Applicants have consistently argued that there is no motivation to combine the references, and that the *combination* of references does not render the invention obvious. Rather than attacking references individually, applicants have discussed individual references either (1) to show that the reference did not contain any explicit motivation to combine the reference with the remaining cited references, or (2) to demonstrate that the Examiner's interpretation of the reference's teaching was incorrect. Particularly in the case of (2), Applicants must be able to explain what the reference in question actually teaches. As the Board will see, the Examiner is merely using rhetoric to mask the deficiencies of the references.

I. The Method Claims Are Not Obvious over the Cited References

1. The Cited References Do Not Teach Every Limitation of the Claims

The combination of prior art references cited by an Examiner, to render any of the claims obvious, must first teach each and every limitation of those claims. *In re Royka*, 490 F.2d 981, 985, 180 U.S.P.Q. 580, 583 (C.C.P.A. 1974); *see also* 2143.03 MANUAL OF PATENT EXAMINING PROCEDURE 2100-128 (2003). Additionally, where an independent claim is nonobvious under 35 U.S.C. § 103(a), then any claim depending therefrom is also nonobvious. 2143.03 M.P.E.P. at 2100-128. The art cited by the Examiner, however, fails to teach every limitation of the claimed invention. In particular, the cited art fails to teach the use of forskolin and the use of the particular combinations of cytokines claimed in the

conditional immortalization and differentiation of human mesencephalon precursor cells, and does not teach the recited monolayer cultures of conditionally-immortalized cells. In arguing that it does, the Examiner has misconstrued the teachings of several of the references. Our reasoning is as follows.

The Examiner cites Hoshimaru *et al.* as teaching the use of forskolin for the differentiation of immortalized neuronal cells into neurons (Advisory Action, at page 3), in a rejection of at least claims 9, 10, and 25-27. The Examiner, however, misconstrues this reference's actual teaching. At page 1522, Hoshimaru states that *previous studies* had found that "several cytokines, or forskolin or growth factors on specific substrates" were needed for differentiation. In contrast, Hoshimaru *et al.* teaches that "suppression of the *v-myc* production is sufficient to differentiate immortalized neuronal progenitor cells into neurons." (*See* page 1521, right column, heading). A reference must be considered as a whole, and portions arguing against or teaching away from the claimed invention must be considered. *Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve, Inc.*, 796 F.2d 443, 448, 230 U.S.P.Q. 416, 419 (Fed. Cir. 1986). Weiss suggests, in passing, the use of forskolin to "influence the differentiation" of precursor cells (see col. 20, lines 46, 51), but does not teach that forskolin may be used to generate *neurons* from the precursor cells. Neither Boss *et al.* nor Prasad *et al.* teach the use of forskolin. Thus, the combination of references cited by the Examiner fails to teach the use of forskolin to cause the differentiation of immortalized neuronal cells into neurons, and cannot be used to reject claims 9, 10 and 25-27 as obvious.

The cited art also does not teach the use of the combinations of cytokines as recited in the claims. In particular, the cited art does not teach the use of GDNF in differentiating the conditionally-immortalized cells, as recited by claims 25-27. In fact, the Examiner nowhere states that the cited art teaches the use of GDNF.

Applicants note that the combination of Hoshimaru *et al.*, Boss *et al.*, and Prasad *et al.* does not teach the combination of EGF, FGF-2 and PDGF in the culture of conditionally-immortalized human mesencephalic progenitor cells, and does not teach the combination of differentiating factors recited in claims 25-27. Thus, these missing teachings must be supplied by Weiss *et al.* for the Examiner's rejection to have even a hint of credibility.

The Examiner cites Weiss *et al.* as teaching "the use of a combination of proliferation inducing growth factors selected from NGF, BDNF, NT-3, NT-4, NT-5, CNTF, FGF-1, FGF-2, EGF, TGFa, TGFb, PDGF, IGFs and interleukins . . . The cited art [*i.e.*,

Weiss *et al.*] further teaches in-vitro proliferation of neuronal progenitor cells in the presence of [the] above mentioned growth factors." Advisory Action at page 3 (emphasis in original).

Although not explicitly stated, the Examiner appears to cite Weiss *et al.*, in combination with the remaining cited art, against method claims 1-5, 9, 10 and 25-27. The Examiner both misconstrues the teachings of Weiss *et al.*, and misapplies those teachings to the claims. At col. 17, lines 1-15, cited by the Examiner, Weiss *et al.* suggests that precursor cells be proliferated in EGF and FGF-2. In contrast, Weiss *et al.* suggests that PDGF *influences differentiation* (col. 17, line 12). According to claim 1 of the instant method, differentiation is strictly controlled by the expression of the oncogene. Moreover, Weiss *et al.* teaches that, after culturing in "a proliferation-inducing growth factor," the disclosed stem cells "begin[] to divide, giving rise to a cluster of undifferentiated cells referred to herein as a 'neurosphere'." (col. 17, lines 17-20). These "neurospheres" are obviously not the monolayer taught by the instant disclosure. Weiss therefore teaches that the combination of EGF and FGF-2 with PDGF has a different *purpose* and achieves a different *result* than in the instant invention. As a result, Weiss *et al.* does not rectify the deficiencies of the remaining cited art, and the combination cannot render the instantly-claimed invention obvious.

Other sections of Weiss *et al.* cited by the Examiner are irrelevant or do not supply the teachings for which the Examiner cites this reference. Col. 22, lines 17-29 teach that various "growth factor products" may be useful in the treatment of CNS disorders. None of the claims of the instant invention are directed to the treatment of CNS disorders. Col. 30, line 17 merely refers to a section heading and provides no useful information. Col. 31, lines 46-64 disclose a list of "biological agents" that may be *tested* to determine their effects on precursor cells (*see* col. 31, lines 29-45). It is clear that the Weiss *et al.* had no idea what the effects of those compounds would be, only that their effects could be tested. Finally, Examples 1-6 teach only the use of EGF in the culture of mouse neural stem cells; Example 7 teaches *differentiation* in EGF-containing medium; and Example 8 teaches the use of CNTF, BDNF or FGF-2 for *differentiation* of the neurospheres. Therefore, Weiss *et al.*, in combination with the remaining cited art, does not teach the culture of conditionally-immortalized human mesencephalon progenitor cells in EGF, FGF-2 and PDGF to produce the cells of the invention, as recited in claim 1.

In sum, Because Hoshimaru *et al.* teaches only the culture in FGF-2 (see page 1519, left column, second full paragraph), Prasad *et* al. teaches only EGF (*see* page 597, right column, first full paragraph, reference 37), Boss *et al.* and Gallyas *et al.* teach *none* of EGF, FGF-2 and PDGF, the combination of these references fails to teach the use of EGF, FGF-2

and PDGF as recited in claim 1 of the instant invention. And since claims 9, 10 and 25-27 depend upon claim 1, the combination of references likewise cannot be used to reject these claims as obvious.

The cited references alone or in combination do not teach the combination of cytokines in the *differentiation* of the conditionally-immortalized human mesencephalon progenitor cells, as recited in claims 25 and 26, respectively, because they fail to teach the use of GDNF. The Examiner concludes that "it would have been further obvious to use a combination of BDNF, CNTF, FGF-2, EGF, PDGF, and IGFs to promote the survival of mesencephalonic dopaminergic neurons in view of Weiss." Advisory Action at page 3 (emphasis in original). Applicants point out that this combination is not claimed. Rather, the Inventors recite the use of a combination of FGF-2, EGF and PDGF in the culture of conditionally-immortalized mesencephalon progenitor cells, and the use of a combination of either forskolin, GDNF and CNTF (claim 26) or forskolin, GDNF, CNTF, IGF-1 and BDNF (claim 27) to differentiate conditionally-immortalized cells *into* neurons. The Examiner's citation of Weiss *et al.* for this point is, therefore, inapt.

Finally, the Examiner, referring to pending claim 1, once again persists in citing Boss *et al.* as teaching "monolayers," in its abstract and at column 11, line 25. Applicants are aware that Boss *et al.* uses the word "monolayer" in the heading of the section that begins at column 11, line 25. However, the Applicants respectfully suggest that the Examiner has ignored *other* statements in Boss *et al.* that indicates that the reference does not actually disclose a "monolayer" as that term is used in the art and in the instant claims. Boss *et al.* specifically states that "[g]ross examination of typical neuron progenitor cell 'monolayer' cultures reveals interconnected three-dimensional structures, rather than the usual two-dimensional monolayer observed with most cell lines."[1] Col. 6, ll. 4-7. Thus, rather than a monolayer of cells, Boss *et al.* actually discloses cells in interconnected *three-dimensional* structures. In contrast, claim 6 of the instant application clearly recites "*adherent* monolayers" - *i.e.,* a two-dimensional layer of cells. The Examiner has still not explained how the clumps of cells disclosed in Boss *et al.* are adherent monolayers. A reference must be cited for what it *fairly* suggests, *In re Burkel*, 592 F.2d 1175, 1179, 201 U.S.P.Q. 67, 70 (C.C.P.A. 1979), and Boss fairly suggests something other than the adherent monolayers recited in claim 1. Boss *et al.* therefore clearly does *not* teach monolayers, as

[1] Indeed, Boss *et al.* here places "monolayer" in quotes to indicate that the cultures are not monolayers as persons of skill in the art recognize them.

recited in claims 1 and 6, and cannot be combined with Hoshimaru *et al.* and/or Prasad *et al.* to render these claims obvious.

Even if one assumes that the Examiner is correct that Boss *et al.* teaches monolayers of precursor cells, the Examiner still fails to consider this reference in light of Weiss *et al.*, which teaches that the culture conditions disclosed therein produce *clusters* of cells, not monolayers. The Examiner, therefore, fails to consider the combined teachings of the references.

In sum, the combination of references cited by the Examiner fails to teach each and every limitation of the claims, as required for a rejection under 35 U.S.C. § 103. The fact that the combination of *five* references cited by the Examiner fails to supply these teachings underscores the non-obviousness of the pending claims. *See, e.g., ATD Corp. v. Lydall, Inc.*, 159 F.3d. 534, 546, 48 U.S.P.Q.2d 1321, 1330 (Fed. Cir. 1998) (invention not obvious over combination of seven references); *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1383, 231 U.S.P.Q. 81, 93 (Fed. Cir. 1986) (invention not obvious over combination of twenty references).

2. The Cited References Do Not Provide a Reasonable Expectation of Success in Practicing the Claimed Invention

The Examiner also fails to establish a reasonable expectation of success in practicing the claimed invention through the combinations of cited art. Our reasoning is as follows.

In order for a combination of references to render a claim obvious, the combination must have provided, at the time of the invention, a reasonable expectation of success in practicing the invention as claimed. *Amgen, Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 1207-08, 18 U.S.P.Q.2d 1022-23 (Fed. Cir. 1991), *cert. denied* 502 U.S. 856 (1991) (holding that to establish obviousness requires the cited references to show that there was, at the time of the invention, a reasonable expectation of success). The Examiner explains that the combination of references would provide a reasonable expectation of success in practicing the claimed invention "because neuronal progenitor cells are easy to transfect, especially in the presence of proliferation enhancing growth factors, which promotes cell survival." Advisory Action, at page 3. This statement, the Examiner's sole basis for a reasonable expectation of success for the majority of the instant claims, is incorrect for several reasons. Contrary to the Examiner's implication, the invention is more than simply transfecting cells; ease of transfection does not mean that a person of skill in the

art would have a reasonable expectation of practicing *the invention* as *claimed*, which encompasses the *transfection* of *human* mesencephalon precursor cells, *culture* of the conditionally-immortalized cells, *differentiation* of these cells, and *culturing* of the neurons obtained thereby.

In making this assertion, the Examiner further presumes that rat cells and human cells are equivalent for the purpose of supporting the cited combination of references. This contention is the Examiner's sole basis for asserting that culture methods useful for rat cells would apply equally well to human cells. However, this contention is incorrect (*see* Section II, below). In fact, human neuronal progenitor cells are expected to respond differently to pharmacologic agonists and antagonists (*see* Sah *et al.*, "Bipotent Progenitor Cell Lines from Human CNS," *Nat. Biotech.* 15(6):574-580 (1997)), and do not proliferate and differentiate in the same manner as rat cells in response to the same culture conditions. Aside from this contention, the Examiner has provided no basis in the cited art—or elsewhere—for the proposition that *human* neuronal progenitor cells would be "easy to transfect," or to culture, or to differentiate.[2] It is apparent, with respect, that the Examiner has come to this belief based on the Inventors' own disclosure, which is improper. *In re Vaeck*, 947 F.2d 488, 493, 20 U.S.P.Q.2d 1438, 1442 (Fed. Cir. 1991) (holding that the reasonable expectation of success must both be found in the prior art, not in the applicant's disclosure).

The assertion of obviousness made by the Examiner is, therefore, essentially that it would be obvious to *try* substituting the rat progenitor cells used in Hoshimaru, *et al.* with human progenitor cells. However, "obvious to try" is an improper basis for a §103(a) rejection. *In re O'Farrell*, 853 F.2d 894, 903, 7 U.S.P.Q.2d 1673, 1680 (Fed. Cir. 1988). Thus, the invention as embodied in the present invention cannot be rendered obvious by the combination of cited references.

3. <u>There is No Motivation to Combine the Cited References</u>

The Examiner has failed to establish that there exists, either within the cited references themselves or in the general knowledge of the art, a motivation to combine the cited art. Our reasoning is as follows.

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[2] The Examiner is called upon to file a Declaration to support the contention that rat neural stem or progenitor cells are equivalent to human neural stem or progenitor cells. Absent this, the rejection is improper.

In order for the combination of references cited by the Examiner to render any claim obvious, there must have been at the time of the invention a motivation to combine the references. *In re Mayne*, 104 F.3d 1339, 1342, 41 U.S.P.Q.2d 1451, 1454 (Fed. Cir. 1997), *In re Jones,* 958 F.2d 347, 351, 21 U.S.P.Q.2d 1941, 1943-44 (Fed. Cir. 1992); *In re Fine*, 837 F.2d 1071, 1075, 5 U.S.P.Q.2d 1596, 1598-99 (Fed. Cir. 1988). The motivation cannot come from the Applicant's disclosure. *In re Fine*, 837 F.2d at 1075, 5 U.S.P.Q.2d at 1599 (obviousness can only be established by combining or modifying the teachings of the prior art to produce the claimed invention where there is some teaching, suggestion or motivation to do so found either in the references themselves or in the knowledge generally available to one of ordinary skill in the art).

The Examiner must provide objective evidence and specific factual findings with respect to the motivation to combine the references. *See In re Lee*, 277 F.3d 1338, 1342-44, 61 U.S.P.Q.2d 1430, 1433-34 (Fed. Cir. 2002). Here, however, the Examiner has provided not the required objective evidence or facts, but only conclusory statements. The Examiner's main argument for combining the cited references is that:

> [o]ne would have been motivated to make immortalized human neuronal progenitor cells wherein the expression of [the]v-*myc* oncogene is driven by [a] tetracycline - controlled tr[a]nsactivator because suppression of [the] v-*myc* oncogene in an immortalized progenitor induces the differentiation of the neuronal progenitor cell. Furthermore, immortalized human neuronal progenitor cells are valuable research tools to understand the molecular mechanism[s] that control the development and function of nervous system cells *in vitro*.

Thus, the Examiner fails to point to any motivation stated within the references themselves that would encourage their combination. This statement, therefore, is legally insufficient to support a rejection for obviousness. *See In re Fine*, 837 F.2d at 1075, 5 U.S.P.Q.2d at 1599.

In fact, there is no motivation within the cited references to combine them. Neither Hoshimaru *et al.* nor Prasad *et al.* teach or suggest the immortalization of human mesencephalon cells. Instead, these references teach the conditional immortalization of *rat* cells; in contrast, neither reference teaches that the method disclosed therein may be used to conditionally immortalize human mesencephalic cells. Hoshimaru *et al.* teaches the conditional immortalization of *rat* cells with tetracycline-regulated *v-myc*. The Examiner states that Prasad *et al.* "teaches that mesencephalic cell[s] could be genetically manipulated." With respect, Applicants argue that this is irrelevant to the instant invention. Prasad *et al.*

does not teach or suggest that the methods disclosed therein are applicable to human cells. Rather, Prasad *et al.* very specifically teaches only that the two SV40 constructs disclosed therein could conditionally immortalize *rat* cells. Boss *et al.* teaches the production of non-immortalized human mesencephalon progenitor cells, but does not suggest the immortalization, conditional or otherwise, of the cells disclosed therein. Thus, there is no motivation to combine Hoshimaru *et al.* or Prasad *et al.* with Boss *et al.* (Weiss *et al.* and Gallyas *et al.* do not teach immortalization.)

The Examiner's statement further fails to explain how the general knowledge of the art would motivate such a combination. For example, the Examiner's statement that the expression of the v-*myc* oncogene induces differentiation fails to explain why such activity would motivate one of skill in the art to combine the cited references. The Examiner further fails to consider that the *only* cited references disclosing immortalized neural cells disclose immortalized *rat* cells. Finally, the Examiner's statement that the claimed cells "are valuable research tools" only points out that the claimed cells were *desirable*, not that a person of skill in the art would have been motivated to combine the references, much less to have the legally requisite expectation of success in obtaining them. However, the desire for a particular result is not a motivation to combine two references; there has to be some teaching in the references themselves or in the art that the references can *be* combined. There is none in the cited references.

The Federal Circuit has stated that "the best defense against the subtle but powerful attraction of a hindsight-based obviousness analysis is rigorous application of the requirement for a showing of the teaching or modification to combine prior art references." *In re Lee,* 277 F.2d at 1343, 61 U.S.P.Q.2d at 1433. This is because "[w]hen prior art references require selective combination by the court to render obvious a subsequent invention, there must be some reason for the combination other than the hindsight gleaned from the invention itself." *Continental Can Co. USA, Inc. v. Monsanto Co.*, 948 F.2d 1264, 1271, 20 U.S.P.Q.2d 1746, 1751 (Fed. Cir. 1991), *Uniroyal, Inc. v. Rudkin-Wiley Corp.*, 837 F.2d 1044, 1051, 5 U.S.P.Q.2d 1434, 1438 (Fed. Cir. 1988) citing *Interconnect Planning Corp. v. Feil*, 774 F.2d 1132, 1143, 227 U.S.P.Q. 543, 551 (Fed. Cir. 1985). The Examiner, with respect, has not applied this requirement "rigorously."

This conclusion is supported, moreover, by the Examiner's failure to take into account the wide variety of methodologies taught by the cited art, as previously pointed out by Applicants in Paper No. 25. A person of skill in the art would not be guided by these various teachings to the practice the methods and cells claimed in the instant application. For

example, the cited references differ in their teachings as to the growth factors to use for proliferation and differentiation. For proliferation, Hoshimaru *et al.* discloses the use of only FGF-2. *See* p. 1519, col. 1, ¶ 3. Weiss *et al.* suggests that cells be proliferated in EGF and FGF-2; that PDGF may influence differentiation (col. 17, line 12); and that CNTF, BDNF or FGF-2 may be used in the differentiation of precursor cells. Prasad *et al.* does not use growth factors. *See* p. 597, col. 2, ¶ 2. Thus, there is no motivation, either within the references or in the art, to combine a reference disclosing the use of several growth factors, a reference disclosing the use of one growth factor, and a reference disclosing the use of *no* growth factors, to arrive at the claimed method.

In another example, Hoshimaru, *et al.* uses DMEM, a minimal medium, and Ham's F-12, a defined medium, with N2 supplement. *See* p. 1519, col. 1, ¶ 3. Prasad, *et al.* uses only the defined medium MCDB-153, which contains different ingredients than DMEM and F-12, followed after one year by F12 medium. *See* p. 597, col. 2, ¶ 2. Furthermore, the culture of the rat cell lines as taught by Prasad, *et al. requires* the use of tissue culture dishes precoated with a specialized substrate, consisting of bovine serum albumen, fibronectin and collagen. *See* p. 597, col. 2, ¶2.

Moreover, while the method of the instant claim 1 recites the selection of a monolayer of precursor cells, the Examiner attempts to combine Boss *et al.*, a reference that (according to the Examiner) teaches the production of monolayers of precursor cells with Weiss *et al.*, which teaches the use of proliferation-inducing growth factors to produce *clusters* of precursor cells. The Examiner fails to explain the motivation for combining these two conflicting teachings; in fact, if the Examiner is correct, these two references *teach away* from each other. Thus, one of skill in the art would not be motivated to combine these references, with the remaining cited art, to arrive at the claimed invention.

The cited art would not be combined to teach the use of forskolin in the differentiation of the conditionally-immortalized human progenitor cells, as the Examiner suggests. Hoshimaru *et al.*, in contrast to the Examiner's characterization teaches that forskolin is *not* necessary for differentiation (*see* Abstract; page 1521, paragraph under heading "Suppression of v-*myc* Production is Sufficient to Differentiate Immortalized Neuronal Progenitor Cells into Neurons"). Weiss *et al.*, in the context of *non*-transfected progenitor cells, suggests (once) the use of forskolin (*see* col. 20, line 51). The remaining cited references do not disclose the use of forskolin. Thus, the *combination* of references teaches away from the use of forskolin when an externally-regulatable oncogene is employed,

as in the current invention. Thus, again, there is no motivation to combine the cited references.

The Examiner has not explained, given the disparate teachings of the cited references, how one of ordinary skill in the art would have determined which elements of the Hoshimaru *et al.*, Prasad, *et al.* or Boss *et al.* teachings to retain, and which to alter, for use with human mesencephalic cells. Neither has the Examiner has pointed to any teaching in the art, or knowledge of one of skill in the art, that would enable such a selection. There is, therefore, no motivation within these references to combine them, or, if combined, to lead one of skill in the art to arrive at the claimed invention. *Compare ATD Corp.*, 159 F.3d at 546, 48 U.S.P.Q.2d at 1330 (no motivation to combine seven references in a crowded field).

Thus, because the motivation to combine the references arise from neither the cited art nor the general teachings of the art, it must have come from the Inventors' own disclosure. It is, of course, improper to use an applicant's disclosure to provide the "hindsight" necessary to combine references that would otherwise not be combined. *In re Vaeck*, 947 F.2d at 493, 20 U.S.P.Q.2d at 1442 (teaching or suggestion to make the claimed combination must be found in the prior art, not in the applicants' disclosure); *In re Deuel*, 51 F.3d 1552, 1558, 34 U.S.P.Q.2d 1210, 1215 (Fed. Cir. 1995). The Examiner cites *In re McLaughlin* in support of the contention that that the Examiner has used no impermissible hindsight to combine the cited references (*see* Paper No. 26, Office Action, mailed September 20, 2002, at page 3). However, given that neither the references nor the art suggests combining the cited references, the Examiner must have used the teachings of the instant specification to combine the cited references. This is improper. *In re Vaeck*, 947 F.2d at 493, 20 U.S.P.Q.2d at 1442; *In re Deuel*, 51 F.3d at 1558, 34 U.S.P.Q.2d at 1215. As such, the references cited by the Examiner should not be combined to reject the instant claims as obvious.

II. The Cell Claims Are Not Obvious over the Cited Art Because Human Cells Are Not Obvious Over Rat Cells

Claims 6-8, 13-15, 23 and 24, directed to conditionally-immortalized cells and neurons differentiated therefrom, are also not obvious over the cited art. In particular, the combination of art disclosing rat neuronal progenitor cells in combination with art disclosing human neuronal progenitor cells does not make the cell claims of the instant application obvious because rat cells are not equivalent to human cells. Our reasoning is as follows.

The Examiner has not made clear what combination of references is being applied to assert the obviousness of the cell claims, but Applicants will assume it is the Hoshimaru *et al.*, Prasad *et al.*, Boss *et al.* and Gallyas *et al.* references. Hoshimaru *et al.*, Prasad *et al.* and Gallyas *et* al. disclose rat cells. The Examiner, explaining for the first time in the latest Advisory Action the basis for equating rat cells and human cells, states that:

> In [the] instant case mammalian mesencephalon neuron progenitor cells (mouse and human) are considered to have identical characteristics, therefore the genetic modification and culturing of human mesencephalon neuron progenitor [cells] with combination [*sic*] of know[n] growth factors is not an unexpected finding especially in view of [the] cited prior art of record.

(Advisory Action at page 2). This is an extraordinary claim, which the Examiner fails to support with any citation or authority. The Examiner certainly does not explain what the "identical characteristics" are that would lead one of skill in the art to equate rat or mouse and human neuronal progenitor cells. In fact, the art of progenitor cell culture does not treat human and rat progenitor cells as equivalent. For example, it is expected that rodent neural cells will behave differently in response to pharmacologic agonists or antagonists. *See, e.g.*, Sah *et al.*, "Bipotent Progenitor Cell Lines from Human CNS," *Nat. Biotech.* 15(6):574-580 (1997).

Applicants point out that rat cells are compositions of matter that are substantially different from human cells. The two come from a completely different source, have different biochemical markers, and react differently to culture and proliferation conditions. Rat cells are considered substantially different than human cells by those in the art. The Examiner has provided no reference suggesting or teaching what modifications of the rat neuronal cells should be performed to arrive at the claimed human neuronal cells. Moreover, rat cells cannot be used for the same purposes as human cells, For example, one would not transplant rat cells into a human patient for treatment purposes because such cells would be readily rejected by the patient's immune system!

The Examiner states that "it would have been obvious . . . to substitute the immortalized rat neuronal progenitor cells as taught by Hoshimaru *et al.* and Prasad *et al.* with human mesencephalon neuron progenitors (Boss *et al.*)." (Advisory Action, at p. 3). This proposed substitution fails because the resulting cells would not be conditionally-immortalized human mesencephalon cells. The Examiner, moreover, fails to explain why, or in what context, such a substitution may be made. The Examiner's statement also fails to

explain how the cell claims of the instant invention are obvious, because the fact that one cell may substitute for another does *not* mean that the second is obvious in light of the first. Applicants note, too, that the Examiner has not suggested that the cells of Boss *et al.* could be conditionally-immortalized.

Applicants respectfully suggest that the Examiner may have confounded the product disclosed in the references with the process used to make them. For example, the Examiner states that "it would have been obvious . . . to substitute the immortalized rat neuronal progenitor cells as taught by Hoshimaru et al and Prasad et al with human mesencephalon neuron progenitor cells as taught by Boss et al." In essence, the Examiner argues that the method of Hoshimaru makes the claimed conditionally-immortalized progenitor cells, and the resulting differentiated cells, obvious. In making this argument, the Examiner follows essentially the same obviousness analysis disallowed in *In re Deuel,* 51 F.3d at 1559, 34 U.S.P.Q.2d at 1216. (method of obtaining a DNA molecule cannot render obvious the DNA molecule itself). Thus, the general method of making immortalized *rat* neuronal progenitor cells taught in Hoshimaru *et al.* cannot render obvious the claimed conditionally-immortalized human neuronal progenitor cell itself.

Thus, the combination of Hoshimaru *et al.*, Prasad *et al.* and Boss *et al.* references do not render the cell of the instant invention, or of claims 6, 13-15, 23 and 24, obvious.

Finally, the Examiner once again rejects claims 7 and 8 in part over Gallyas *et al.*, because the Examiner believes that the reference teaches the characterization of mouse or rat immortalized neuronal cell lines by measuring the concentration of various neurotransmitters such as GABA and dopamine. Gallyas *et al.* is irrelevant to claim 7 and claim 8 because neither of these claims recites *methods* for identifying GABAergic or dopaminergic neurons. Instead, the claims are directed to conditionally immortalized *cells* that can *differentiate* into neurons that are GABAergic or dopaminergic. Applicants respectfully restate that the Examiner cites Gallyas *et al.* for the wrong proposition; thus the reference cannot be used in combination with any other cited reference to reject claims 7 and 8.

5. The Invention Satisfies a Long-Felt Need

Finally, Applicants point out that they were the first to produce conditionally-immortalized *human* mesencephalic cells, and to differentiate them into mature mesencephalic neural cells. This accomplishment satisfied a long-felt need in the art because

they would be "valuable research tools," as the Examiner has stated. *See, e.g.*, Lotharius *et al.*, "Effect of Mutant α-Synuclein on Dopamine Homeostasis in a New Human Mesencephalic Cell Line," *J. Biol. Chem.* 277(41):38884-94 (2002) (submitted herewith as Exhibit 7). However, the primary art cited by the Examiner against the present invention dates to more than *two years* prior to Applicants' filing date; the Hoshimaru *et al.* reference published in February 1996 (reporting work that had been done in 1995), and the Boss *et al.* reference claims priority to an application filed in 1989. In that time, no other parties were able to generate conditionally-immortalized human mesencephalon cells. The Examiner has failed to explain why, if the cited references render the invention obvious, and the claimed cells and methods were so valuable, no other persons of skill in the art were able to develop these cells and methods prior to the Inventors. The clear reason is that the present invention is not, in fact, obvious, and its execution far less straightforward and routine than the Examiner believes it was at the time of filing.

CONCLUSION

Thus, for the reasons enumerated above, Applicants believe that the instantly-claimed invention is not, in fact obvious over the cited art. Applicants therefore respectfully request the Board to overturn the Examiner's determination that the claims are unpatentable as obvious.

Respectfully submitted,

Attorney for Appellants

Date: November 7, 2003

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Attachments

Exhibit 1

Claims on Appeal

1. A method for producing a conditionally-immortalized human mesencephalon neural progenitor cell, comprising:

(a) plating human mesencephalon cells on a first surface and in first growth medium that permits proliferation;

(b) transfecting said progenitor cells with DNA encoding a selectable marker and an externally regulatable growth-promoting protein; and

(c) selecting an adherent monolayer of the transfected cells on a second surface and in a second serum-free growth medium that permits attachment and proliferation, wherein the second serum-free growth medium comprises FGF-2, EGF and PDGF, and therefrom producing a conditionally-immortalized human mesencephalon cells in which the growth-promoting protein is regulated by an external factor, such that suppression of the growth promoting protein results in differentiation of the cell into a neuron.

2. The method of claim 1 wherein the first and second surfaces are independently selected from the group consisting of substrates comprising one or more of a polyamino acid, fibronectin, laminin or tissue culture plastic.

3. The method of claim 1 wherein the growth-promoting gene is an oncogene.

4. The method of claim 3 wherein the oncogene is v-myc.

5. The method of claim 1 wherein expression of the growth-promoting gene is inhibited by tetracycline.

6. A conditionally-immortalized human mesencephalon neural progenitor cell capable of differentiation into neurons, wherein the cell is transfected with DNA encoding a growth-promoting protein that is regulated by an external factor, such that suppression of the growth-promoting protein results in differentiation of the cell into a neuron, and wherein the cell is polygonal and grows as an adherent monolayer.

7. A conditionally-immortalized human mesencephalon neural precursor cell according to claim 6, wherein the cell is capable of differentiation into dopaminergic neurons.

8. A conditionally-immortalized human mesencephalon neural precursor cell according to claim 6, wherein the cell is capable of differentiation into GABA-ergic neurons.

9. A method for producing a neuron, comprising culturing a cell produced according to claim 1 in the presence of at least one differentiating agent under conditions that inhibit expression of the growth-promoting gene.

10. A method according to claim 9, wherein the cell is cultured in medium comprising tetracycline.

13. A neuron produced according to the method of claim 9.

14. A dopaminergic neuron produced according to the method of claim 9.

15. A GABA-ergic neuron produced according to the method of claim 9.

23. A conditionally-immortalized human mesencephalon neural precursor cell produced according to the method of claim 1.

24. A cell according to claim 23, wherein the cell is present within a clonal cell line.

25. The method of claim 9, wherein the differentiating agent comprises the combination of forskolin, GDNF and CNTF.

26. The method of claim 9, wherein the differentiating agent comprises the combination of forskolin, GDNF, CNTF, IGF-1 and BDNF.

27. The method of claim 9 wherein said differentiating agent comprises GDNF.

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Neurobiology

Differentiation of the immortalized adult neuronal progenitor cell line HC2S2 into neurons by regulatable suppression of the v-*myc* oncogene

(retroviral vector/tetracycline/electrophysiology)

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ABSTRACT **A regulatable retroviral vector in which the v-*myc* oncogene is driven by a tetracycline-controlled transactivator and a human cytomegalovirus minimal promoter fused to a *tet* operator sequence was used for conditional immortalization of adult rat neuronal progenitor cells. A single clone, HC2S2, was isolated and characterized. Two days after the addition of tetracycline, the HC2S2 cells stopped proliferating, began to extend neurites, and expressed the neuronal markers tau, NeuN, neurofilament 200 kDa, and glutamic acid decarboxylase in accordance with the reduced production of the v-*myc* oncoprotein. Differentiated HC2S2 cells expressed large sodium and calcium currents and could fire regenerative action potentials. These results suggest that the suppression of the v-*myc* oncogene may be sufficient to make proliferating cells exit from cell cycles and induce terminal differentiation. The HC2S2 cells will be valuable for studying the differentiation process of neurons.**

Much of our understanding of the molecular mechanisms that control the development and the function of nervous system cells has been derived from studies of cells in culture. Clonal cultures of progenitor cells are useful for studying aspects of th differentiation pathways leading to the formation of mature neurons or glia. Primary cultures of progenitor cells, being heterogeneous in nature, do not offer such an opportunity. As an alternative approach, neural cell lines have been generated by various techniques (1–5). One common approach has been to immortalize cells by transducing neuroepithelial or neural progenitor cells from developing brain with retroviral vectors encoding the simian virus 40 (SV40) large tumor (T) antigen or *myc* oncogenes (1–12). Studies have shown that the immortalization process arrests cells at specific stages of development and prevents their terminal differentiation (9). As a result, cells at specific stages in the development can be immortalized and used to study the nature and potentiality of the cells at this particular stage in the lineage and how they can be further differentiated down that particular pathway.

In neural cell lines developed by constitutively expressing oncogenes such as *myc*, the mitotic activity of the oncogene is always present, and cells proliferate continuously in culture (6, 9, 13). Differentiation of the immortalized progenitor cells into neurons may require sufficient down-regulation of the oncogene, but attempts to induce such terminal differentiation often induce apoptosis (14). However, partial differentiation f c-*myc*-immortalized cells has been achieved by treatment with growth factors (6). To obtain a regulatable expression of the oncogen , a temperature-sensitive mutant of SV40 large T antigen (tsA58) has been used for conditional immortalization (3, 7, 8, 10–12, 15). At a permissive temperature when the large T antigen is expressed, the immortalized cells are undifferentiated and are multipotent in nature (3, 7, 8), as has been seen with *myc*-immortalized cells (6). Although at nonpermissive temperature the expression of T antigen is considerably down-regulated and the cells are not under the mitotic drive of the oncogene, the temperature shift results in only incomplete differentiation of cells into neurons (7, 8, 11, 12, 15). A combination of factors and substrates is needed to further differentiate the cells *in vitro* (12). A simpler system in which the regulatable suppression of oncogene expression in immortalized neuronal progenitor cells could allow their differentiation into neurons without the help of complex factors and substrates would be useful.

To build a system in which the oncogene expression can be regulated by exogenous agents, we have taken advantage of a tetracycline-controlled gene expression system (16–19). In this system, a tetracycline-controlled transactivator (tTA), which is a fusion protein of the repressor (*tetR*) of the Tn*10*-derived tetracycline-resistance operon of *Escherichia coli* and the acidic domain of VP16 of herpes simplex virus, strongly activates transcription from $P_{hCMV^{*}\text{-}1}$, a minimal promoter from human cytomegalovirus (hCMV) fused to the tetracycline (*tet*) operator sequences in the absence of tetracycline. Low concentrations of tetracycline (0.01–1.0 μg/ml), at which no toxic effect is evident, almost completely abolishes transcription activation by tTA (16, 17). Using this vector system, we constructed a retroviral vector (LINXv-*myc*) in which the v-*myc* oncogene is transcribed in a tetracycline-regulated fashion. Neural progenitor cells cultured from adult rat hippocampus were transduced with the retroviral vector LINXv-*myc*, and a stably transfected colony was isolated and grown. Here we report that tetracycline suppresses the production of v-*myc* and allows the progenitor cells to terminally differentiate into neurons. Since the progenitor cell line differentiates into neurons only, we may have immortalized a cell that has already been committed to a neuronal lineage. This cell line will be useful for studying the mechanisms of cell differentiation *in vitro*.

EXPERIMENTAL PROCEDURES

Vector Construction. The retroviral vector LINXv-*myc* was constructed as follows: the 1.02-kb *Eco*RI–*Bam*HI fragment of the tTA was excised from pUHD15-1 (16) and inserted into pHENA (20) just upstream of the internal ribosome entry sit (IRES) of the encephalomyocarditis virus. The resultant 2.78-kb *Eco*RV–*Bam*HI fragment containing tTA, IRES, and

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Abbreviations: FGF-2, basic fibroblast growth factor; tTA, tetracycline-controlled transactivator; IRES, internal ribosomal entry site; GAD, glutamic acid decarboxylase; GFAP, glia fibrillary acidic protein; SV40, simian virus 40; T, tumor; LTR, long terminal repeat; NFH, neurofilament 200 kDa.

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neomycin phosphotransferase was inserted into the polylinker site (*Hap* I and *Bam*HI) of LXSHD (21). Then, the portion of LXSHD spanning the SV40 early promoter and histidinol dehydrogenase cDNA was replaced by a $P_{hCMV*-1}$-v-*myc* (3.32 kb) fragment. $P_{hCMV*-1}$ was derived from pUHD10-3, and a 2.87-kb *Sac* I–*Sph* I fragm nt of v-*myc* was derived from pMC38 (American Type Culture Collection).

Cell Culture. All packaging and producer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. ψ-2 cells were transfected with 10 μg of LINXv-*myc* plasmid DNA by the calcium phosphate procedure. Virus-containing medium collected from ψ-2 cells 2 days after transfection was used to infect the amphotropic PA317 cells. One day after the infection, the cells were split at a 1:10 ratio, plated, and selected for G418 (400 μg/ml) resistance. Colonies were picked after selection for 8 days and tested for the proper integration of the vector, production of v-*myc*, and the titer of the retrovirus.

Cells from adult (3-month-old) rat hippocampus were isolated and cultured as described (22) with the following modifications. Cells isolated from tissue after enzymatic dissociation were resuspended in DMEM/Ham's F-12 (1:1, vol/vol) high glucose medium (Irvine Scientific) containing 10% fetal bovine serum and then plated onto uncoated plastic tissue culture flasks (1×10^6 cells per 75 cm^2 flask). The cells were grown at 37°C in a 5% CO_2 incubator. The next day, the serum-containing medium was replaced with serum-free DMEM/Ham's F-12 medium containing N-2 supplement (insulin at 5 μg/ml, human transferrin at 50 μg/ml, 20 nM progest rone, 100 μM putrescine, 30 nM sodium selenite, 2.5 mM glutamine; GIBCO) and basic fibroblast growth factor (FGF-2; human recombinant) at 20 ng/ml. Confluent cultures f cells were passaged to polyornithine/laminin-coated plates and cultured. Proliferating cultures, maintained for about a year through 19 passages were split at a 1:3 ratio and 1 day later infected for 20 hr with a mixture of one volume of the conditi ned medium of the producer cell line and two volumes of DMEM/Ham's F-12 containing N-2 supplements, FGF-2 at 20 ng/ml, and Polybrene at 4 μg/ml. The infected cells were split at a 1:4 ratio and selected in the presence of G418 (100 μg/ml).

Northern Blot Analyses. Total RNA was isolated by the CsCl/guanidinium thiocyanate method (23). Fifteen micrograms of total RNA was separated on formaldehyde/agarose (1.5%) gels and transferred onto a Magnagraph nylon membrane and probed with randomly primed v-*myc* or cyclophilin (pB1B15).

Immunofluorescence Staining. Cells were plated in the absence or presence of tetracycline (1 μg/ml) onto Lab-Tek glass chamber slides (Nunc) coated with polyornithin/laminin and cultured for 1 (without tetracycline) or 5 (with tetracycline) days and then fixed for 10 min with 4% paraformaldehyde. The cells were incubated sequentially with the primary antibody in PBS containing 4% donkey serum and 0.3% Triton X-100 overnight at 4°C followed by fluorescein isothiocyanate-conjugated secondary antioody (Jackson Immunoresearch Laboratories; used at 1:500) for 4 hr at room temperature. Slides were mounted in 24% glycerol and 9.6% poly(vinyl alcohol) containing 2.5% 1.4-diazobycyclo[2.2.2]octane. The monoclonal antibodies used were to tau (Boehringer Mannheim; used at 1:250), neurofilament 200 kDa (NFH) (clone RT97; Boehringer Mannheim; used at 2 mg/ml), and NeuN (used at 1:5; ref. 24); polyclonal antibodies used were to vimentin (Amersham; used at 1:10), glial fibrillary acidic protein (GFAP) (Chemicon; used at 1:2000), nestin (used at 1:10,000), and v-*myc* (Upstate Biotechnology; used at 1:8000). For immun fluorescent staining for bromodeoxyuridine (BrdUrd), cells were incubated with BrdUrd (Amersham; used at 1:1000) for 18 hr, fixed with 95% ethanol/5% acetic acid, and then incubated with m noclonal anti-BrdUrd antibody (Amersham; undiluted). Immunoreactivity was detected with anti-mouse biotinylated IgG f llowed by streptavidin-Texas Red conjugate.

Confocal microscopic images of cells were obtained using a Bio-Rad MRC600 confocal microscope equipped with a krypton/argon laser and coupled to a Zeiss Axiovert microscope. Images were collected sequentially using the K1/K2 filter blocks matched to the appropriate excitation filter for each channel. Transmitted light images of differential interference contrast optics were captured in registration with the fluorescent signals using the transmitted field detector. Instrument settings to preclude background fluorescent signals were set against a negative control well where the primary antibodies had been omitted; gain and black levels were adjusted to preclude any signal from this well before imaging other wells for positive signal. Collected digital images were composited in ADOBE PHOTOSHOP 3.0 and printed on a Fujix Pictrography 3000. Phase-contrast images were photographed on a Nikon Diaphot using T-Max film and the negatives digitized using a Leaf Lumina. These images were printed as described above.

Reverse PCR Analysis. One hundred nanograms of total RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase (Promega) with random hexamers (10 μM) as primers in a 20-μl reaction mixture containing 10 mM Tris Cl (pH 8.4), 50 mM KCl, 3.8 mM $MgCl_2$, 1 mM each dATP, dTTP, dCTP, and dGTP, and 20 units of RNasin (Promega). After 75 min at 42°C, the reaction was terminated by heat inactivation at 95°C for 5 min. For PCR amplification, several sets of specific oligonucleotide pairs (10 ng/μl) were incubated with the above reaction mixture and 5 units of *Taq* polymerase (Perkin–Elmer) in a 100-μl reaction mixture containing 10 mM Tris Cl (pH 8.4), 50 mM KCl, 1 mM $MgCl_2$, and 2 μCi of [α-^{32}P]dCTP (1 Ci = 37 GBq). Cycle parameters were 2 min at 94°C, 2 min at 60°C, and 2 min at 72°C for 23 cycles followed by a final 10-min incubation at 72°C. Forty microliters of each reaction was analyzed by electrophoresis on 8% polyacrylamide gels followed by autoradiography. Control experiments using primers for the rat ribosomal protein L27a (internal control) showed that the amount of amplified PCR product was directly proportional to the amount of input RNA after 23 cycles of amplification (data not shown). The sequences of genomic DNA between two primers were selected so that they contained one or two introns for discrimination between products from RNA and contaminating genomic DNA. The following oligonucleotides were used as primers (nucleotide positions are shown in parentheses): rat ribosomal protein L27a (25) 5′ primer: 5′-ATCGGTAAGCACCGCAAGCA-3′ (69–88), 3′ primer: 5′-GGGAGCAACTCCATTCTTGT-3′ (302–283); rat GFAP (26) 5′ primer: 5′-ACCTCGGCACCCTGAGGCAG-3′ (459–478), 3′ primer: 5′-CCAGCGACTCAACCTTCCTC-3′ (599–580); rat glutamic acid decarboxylase (GAD) (27) 5′ primer: 5′-AAGGTTTTGGACTTCCACCAC-3′ (589–609), 3′ primer: 5′-CATAAGAACAAACACGGGTGC-3′ (855–835); rat NFH (28) 5′ primer: 5′-GAGGAGATAACTGAGTACCG-3′ (247–266), 3′ primer: 5′-CCAAAGCCAATCCGACACTC-3′ (548–529).

Electrophysiology. The whole-cell configuration of the patch clamp technique was used to study voltage-gated currents. Pipettes (3- to 5-MΩ resistance) were pulled from Boralex glass (Rochester Scientific), coated with Sylgard (Dow Corning Corp.) and fire-polished. They were filled with internal solution c ntaining 108 mM cesium methanesulfonate, 4 mM $MgCl_2$, 9 mM EGTA 9 mM Hepes, 4 mM ATP, 14 mM creatine phosphate (Tris salt), 0.3 mM GTP (Tris salt), and creatine phosphokinase (50 units/ml) at pH 7.4 with CsOH. Whole-cell recordings were initially established in the bath solution [Tyrode's: 150 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes (pH 7.4 with NaOH), 2 mM $CaCl_2$ and, in some cases, 4 mM $BaCl_2$ added]. Sodium currents were characterized in this bath solution, whereas calcium currents

were assessed with external solution containing 160 mM tetraethylammonium chloride, 10 mM $BaCl_2$, and 10 mM Hepes (pH 7.4) with tetraethylammonium hydroxide and 1 μM tetrodotoxin (Sigma) added. External solutions flowed from an array of microcapillary tubes (internal diameter, 140 μm), driven by gravity; solution exchange was complete in less than 500 msec.

Whole-cell currents were recorded using an Axopatch 200A patch clamp amplifier and the BASIC-FASTLAB interface system (Indec Systems, Santa Cruz, CA). Voltage-dependent currents were filtered at 10 kHz (4-pole Bessel low-pass) and digitized every 25 μs (sodium current) or 50 μs (calcium current). Series resistance compensation was employed, typically for 80–95% of the series resistance measured from the uncompensated capacity transient (dividing the decay time constant by cell capacitance) or from the potentiometer used for nulling the capacity transient. Data were accepted for sodium and calcium current only if the remaining voltage error (calculated as the current times the uncompensated series resistance) was <1 mV and if voltage control was adequate as judged by a graded increase in peak current as test depolarizations were increased. Reported potentials have been corrected for a liquid junction potential of −10 mV between the internal solution and the Tyrode's solution in which the pipette current was zeroed before sealing onto the cell. Sodium and calcium channel currents were corrected for leak and capacitative currents by subtraction of an appropriately scaled current elicited by a hyperpolarization from −80 mV to −90 mV. All experiments were done at 21–25°C. Statistics are given as the mean ± SEM.

RESULTS

Design of the Vector and Generation of the Producer Cell Line. The control elements of tetracycline-resistance operon encoded in *E. coli* Tn*10* have been used to generate a tetracycline-regulatable vector system (16, 17). In this vector the prokaryotic *tet* repressor was converted to a eukaryotic transactivator by fusion of the repressor with the activating domain (C-terminal) of herpes simplex virus VP16 protein. This transactivator strongly activates transcription from the minimal promoter P_{hCMV^*-1} fused to *tet* operator sequences (16). Although this promoter has very low basal activity in HeLa cells or in most tissues of transgenic mice, the synthesis of tTA activates the P_{hCMV^*-1} promoter (16–19). Using this vector system, we constructed a retroviral vector to express the v-*myc* oncogene from the P_{hCMV^*-1} promoter in a tetracycline-regulated fashion (Fig. 1*A*). The long terminal repeat (LTR) of Moloney murine sarcoma virus transcribed a 7.8-kb mRNA

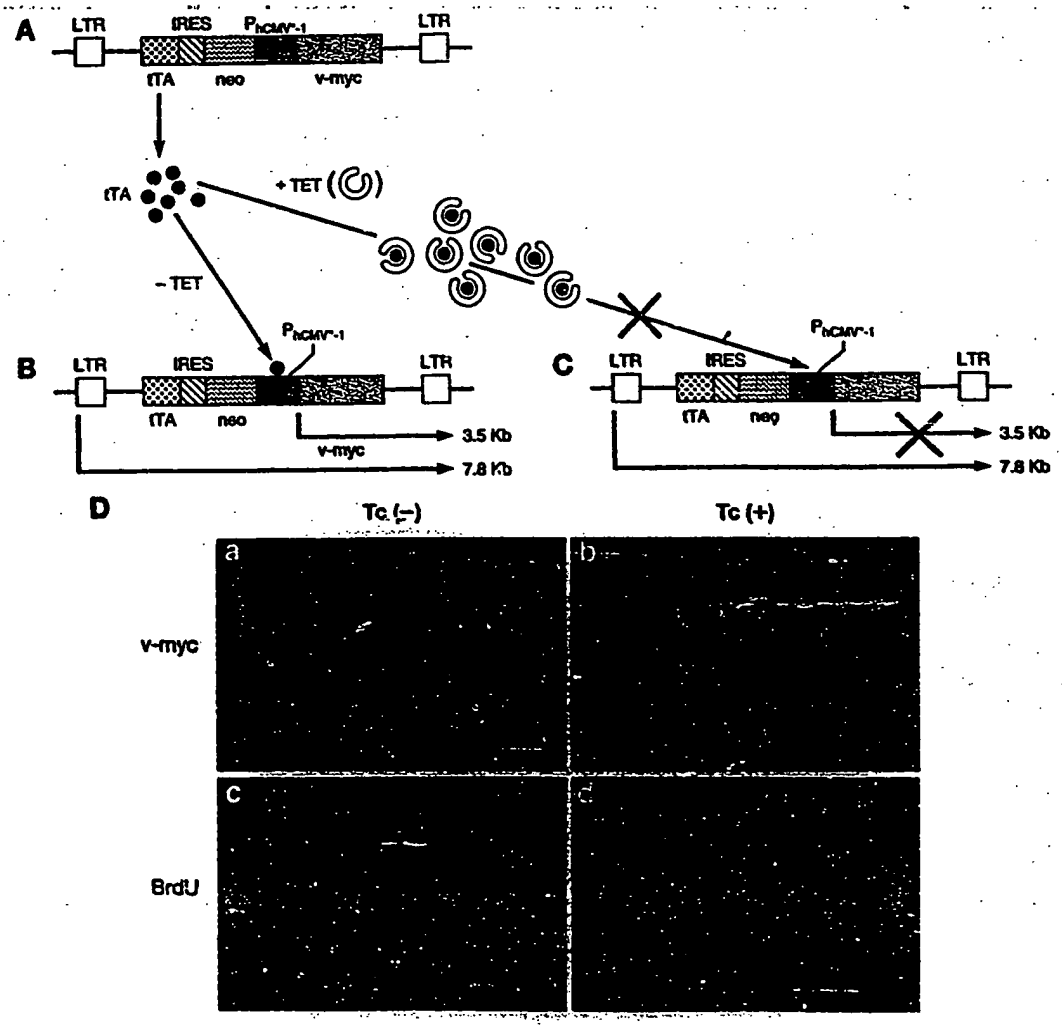

FIG. 1. Structure of LINXv-*myc*. (*A*) The LTR transcribes the 7.8-kb mRNA, which produces two proteins: tTA and neomycin phosphotransferase (neo), with the assistance of the encephalomyocarditis virus IRES. (*B*) In turn, tTA activates the tTA-dependent promoter (P_{hCMV^*-1}) and transcribes the 3.5-kb mRNA, which produces the v-*myc* oncoprotein. (*C*) Tetracycline inhibits the ability of tTA to transcribe the 3.5-kb mRNA. (*D*) Immunofluorescence staining of HC2S2 cells for v-*myc* and BrdUrd. The HC2S2 cells were grown in the absence of tetracycline [Tc(−)] for 1 day (*a* and *c*) and in the presence of tetracycline [Tc(+)] (1 μg/ml) for 2 days (*d*) or 3 days (*b*). The cells were stained with anti-v-*myc* antibody (*a* and *b*) or incubated with BrdUrd for 18 hr and stained with anti-BrdUrd antibody (*c* and *d*).

containing both tTA and ne mycin phosphotransferase genes by means of an IRES (Fig. 1*B*). The tTA binds to *tet* perat r sequences present in the hybrid promoter in the absence of tetracycline and stimulated transcription from $P_{hCMV*-1}$ t yield a 3.5-kb mRNA, which produced the v-*myc* ncoprotein (Fig. 1*B*). Tetracycline (1 μg/ml) suppressed the function of the tTA almost completely and inhibited the production of v-*myc* (Fig. 1*C*). An amphotropic producer cell line producing LINXv-*myc* retrovirus was selected and expanded. The virus had a titer of 10^5 colony-forming units per ml for NIH 3T3 cells and did not contain helper viruses.

Is lation of Immortalized Neuronal Progenitor Cells. Cultured adult rat hippocampal cells were infected with the LINXv-*myc* retrovirus and selected for stable transfectants. One culture consisting of several colonies, HC2, showed some cells with neuronal morphology 3 days after the addition of tetracycline at 1 μg/ml. HC2 cells were plated at low density (1:1000), and a single colony was isolated (HC2S2).

The HC2S2 cells were polygonal and had very small processes (Fig. 2*A*). Southern blot analysis showed that these cells had a single retroviral genome integration site and therefore can be considered a single clone (data not shown). The HC2S2 cells grew very rapidly (doubling time of 12 hr) and were not c ntact inhibited for growth. However, 2 days after the additi n of tetracycline at 1.0 μg/ml, the cells stopped dividing. Most of the cells became phase-bright and started to extend processes, which began to be interconnected by 3 days after the addition of tetracycline (Fig. 2*B*). BrdUrd incorporation studies showed that, when grown in the absence of tetracycline, essentially all HC2S2 cells incorporated BrdUrd (Fig. 1*Da*). However, tetracycline treatment for 2 days followed by incubation with BrdUrd resulted in only 1–2% of the cells incorporating the label (Fig. 1*Dd*).

Northern blot analysis showed the production of large amounts of 3.5-kb v-*myc* transcript in HC2S2 cells grown in the absence of tetracycline. However, amounts of the transcript were reduced after the addition of tetracycline. These results suggest that tetracycline can regulate the transcription of v-*myc* from the hybrid promoter in the HC2S2 cells (Fig. 2*C*). The transcription from the LTR promoter was also reduced (Fig. 2*C*). At the protein level, the expression of v-*myc* oncoprotein in the nucleus of the HC2S2 cells was also down-regulated after the addition of tetracycline (Fig. 1 *Da* and *Db*). Although the suppression of v-*myc* production by tetracycline was enough to st p proliferation of the HC2S2 cells and induce differentiation, the suppression of the oncogene was not complete.

Suppression of th v-*myc* Production Is Sufficient to Differentiate Immortalized Neuronal Progenitor Cells into Neurons. The expression of the neural precursor cell markers nestin and vimentin and the neuronal markers tau, NeuN, and NFH in HC2S2 cells under proliferative and differentiating conditions was examined. When grown in the absence of tetracycline, cells expressed nestin and vimentin (data not shown). Although a few cells were cytoplasmically stained by the anti-NFH antibody (Fig. 3*A*), no staining of cells by the anti-tau or anti-NeuN antibodies was observed (Fig. 3 *C* and *E*). Five days after the addition of tetracycline, almost all cells in culture became positive for NFH, and over half of the cells became positive for tau and NeuN (Fig. 3 *B*, *D*, and *F*). Cell bodies as well as processes were stained by the anti-NFH and anti-tau antibodies (Fig. 3 *B* and *D*), and nuclei were stained by the anti-NeuN antibody (Fig. 3*F*). However, nestin and vimentin staining was no longer present in the cells (data not shown).

In parallel to the immunological studies, PCR analyses showed the expression of differentiated neural cell markers by HC2S2 cells. Designs for primers predicted PCR products of 234 bp for the ribosomal protein L27a, 267 bp for the adult type of GAD, 353 bp for the embryonic type of GAD, 141 bp for GFAP, and 302 bp for NFH. The primer design for GAD can discriminate between the embryonic type of GAD, which is a truncated inactive enzyme, and the adult type of GAD, which is an active enzyme (29). The PCR study demonstrated a marked increase of both types of GAD mRNA in the differentiated HC2S2 cells 3 days after the addition of tetracycline (Fig. 4). A very weak NFH PCR product was detected in the HC2S2 cells grown without tetracycline and paralleled the finding that only a few cells were immunoreactive for anti-NFH. However, there was an increase of the NFH PCR product after the addition of tetracycline (Fig. 4). GFAP mRNA could not be detected in cells grown with or without tetracycline (Fig. 4).

Immortalized Cells Express Sodium and Calcium Currents After Suppression of v-*myc* Production. Electrophysiological

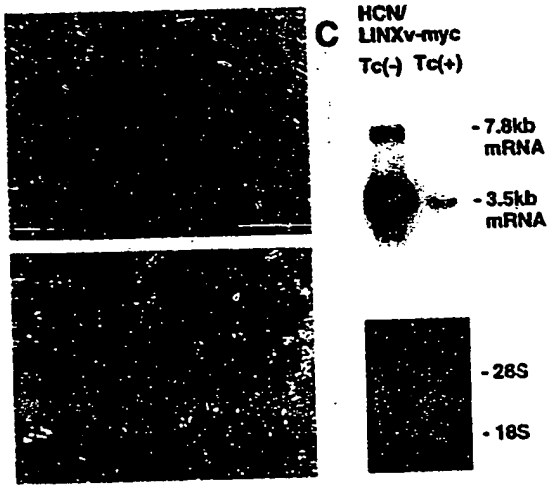

FIG. 2. The cultures derived from a single clone (HC2S2) were grown in the absence of tetracycline for 2 days (*A*) or in the presence of tetracycline (1 μg/ml) for 3 days (*B*). (*C*) The Northern blot analysis shows the transcripts from the LTR (7.8-kb mRNA) and the transcripts from $P_{hCMV*-1}$ (3.5-kb mRNA) in the cells grown in the absence [Tc(−)] or presence [Tc(+)] of tetracycline. The photograph of the gel stained with ethidium bromide is presented as an internal control.

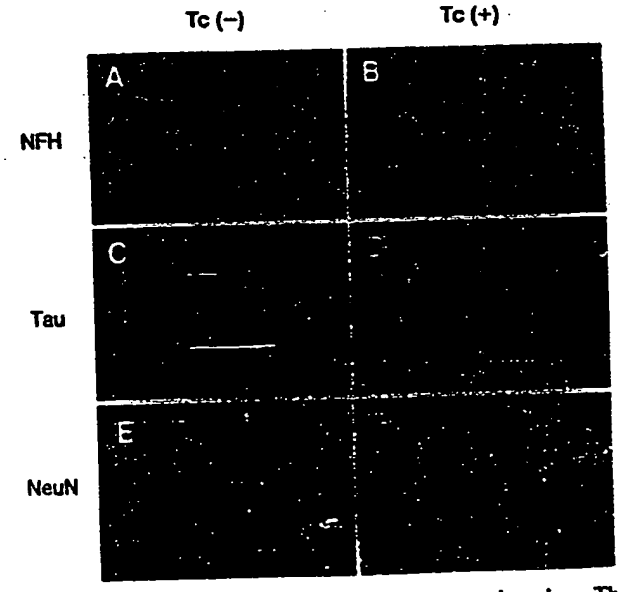

FIG. 3. Immunofluorescence staining for neuronal markers. The cells were grown in the absence of tetracycline [Tc(−)] for 1 day (*A*, *C*, and *E*) or in the presence of tetracycline [Tc(+)] (1 μg/ml) for 5 days (*B*, *D*, and *F*). The cells were stained with anti-NFH antibody (*A* and *B*), anti-tau antibody (*C* and *D*), and anti-NeuN antibody (*E* and *F*).

rec rdings from HC2S2 cells after growth in proliferative and differentiating conditions provided additional evidence that differentiated HC2S2 cells represent neurons (Fig. 5). HC2S2 cells were examined with whole-cell voltage clamp after growth in proliferative (FGF-2, −Tc) and differentiating (FGF-2, +Tc for 6 days) conditions. In proliferative conditions, cells were quiescent, exhibiting virtually no sodium (5 ± 1 pA/pF; 12 cells) or calcium (4 ± 1 pA/pF; 11 cells) current. After growth with tetracycline, substantial sodium and calcium currents were present (Fig. 5 *A* and *B*; +Tc). The densities of restored sodium and calcium currents (Fig. 5*C*) were 96 ± 18 pA/pF (16 cells) and 21 ± 3 pA/pF (10 cells), respectively, which is similar to those present in primary, differentiated fetal rat hippocampal cells in culture (D.W.Y.S., unpublished observations). Sodium and calcium currents were activated at potentials more positive than −40 mV, peaked at −10 ± 2 mV (10 cells) and −3 ± 2 mV (4 cells), and reversed at 78 ± 2 mV (8 cells) and 55 ± 9 mV (3 cells), respectively. The restored sodium current in differentiated HC2S2 cells was of the neuronal form, exhibiting a voltage dependence of inactivation with a midpoint of −60 ± 1 mV (14 cells) and rapid kinetics (time-to-peak of 0.8 ± 0.1 msec; 13 cells). In addition, the restored sodium current was blocked by 1 μM tetrodotoxin (95% ± 1% inhibition, 6 cells). Concomitant with sodium current induction, HC2S2 cells acquired the ability to fire regenerative action potentials after differentiation with tetracycline (data not shown).

DISCUSSION

Advances in cell and molecular biology are predicated on reliable and versatile *in vitro* systems. The paucity of brain-derived neuronal cell lines that can differentiate into mature neurons *in vitro* is a hindrance to a more complete understanding of the central nervous system at cellular and molecular levels. The temperature-sensitive variant of SV40 large T antigen (tsA58) has been the main choice for the conditional immortalization of the neuronal progenitor cells (7, 8, 10–12, 15). However, most of the studies have failed to demonstrate full differentiation of the immortalized cells by simply switching the temperature from a permissive to a nonpermissive one (7, 8, 11, 12, 15). The presence of several cytokines, or forskolin or growth factors on specific substrates, in addition to shifting the temperature, is needed for the differentiation of immortalized precursor cells (11, 12). Although some of these cell lines express NFH after differentiation, the precursor cell marker nestin is still expressed in them (8, 15). These results may indicate that, although the cells express the marker for differentiated neurons, cells are still in the precursor state and not fully differentiated. A simpler system in which the production of oncoprotein can be regulated by exogen us agents to induce differentiation of the progenitor cells will be useful.

Recently, the bacterial tetracycline-resistance operon regulatory system from *E. coli* Tn*10* has been used to develop a tetracycline-responsive promoter system for eukaryotic cells (16, 17). The system functions well in mammalian cells (16) and has been used to generate transgenic mice (18, 19). Transgene expression can be temporally regulated in most tissues of the animal by providing tetracycline exogenously (19). The vector has also been used to control SV40 large T antigen expression and conditional transformation of pancreatic β cells in transgenic mice (18). In this experimental system, the expression of T antigen can be inhibited *in vivo* by providing tetracycline to the animals, but the oncoprotein expression can be restored by removal of the drug. We constructed a retroviral vector in which the expression of v-*myc* oncogene is transcribed by the tetracycline-responsive binary system. The transcription of v-*myc* from the hybrid $P_{hCMV*-1}$ promoter in hippocampal neuronal progenitor cells was found to be efficient and strong enough to immortalize these cells. Although the down-regulation of v-*myc* production by tetracycline was enough to stop proliferation of the HC2S2 cells and induce differentiation, the suppression of v-*myc* mRNA and protein was not complete. To date, this vector has only been used to immortalize progenitor cells from adult rat hippocampus. It will be important to immortalize progenitor cells from other brain regions to examine the regional specificity of the differentiation process.

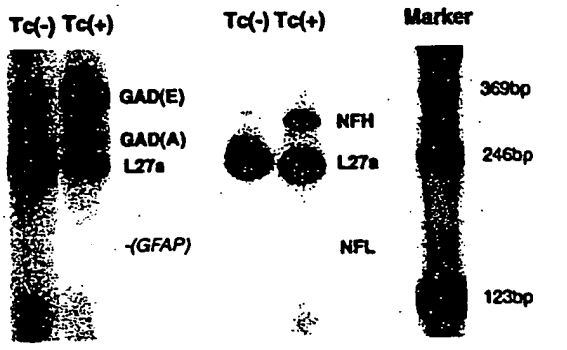

FIG. 4. PCR products of embryonic (E) type of GAD (353 bp), adult (A) type of GAD (267 bp), ribosomal protein L27a (234 bp), GFAP (141 bp), and NFH (302 bp) detected in the cells grown in the absence [Tc(−)] or presence [Tc(+)] of tetracycline. NFL, neurofilament 68 kDa (155 bp).

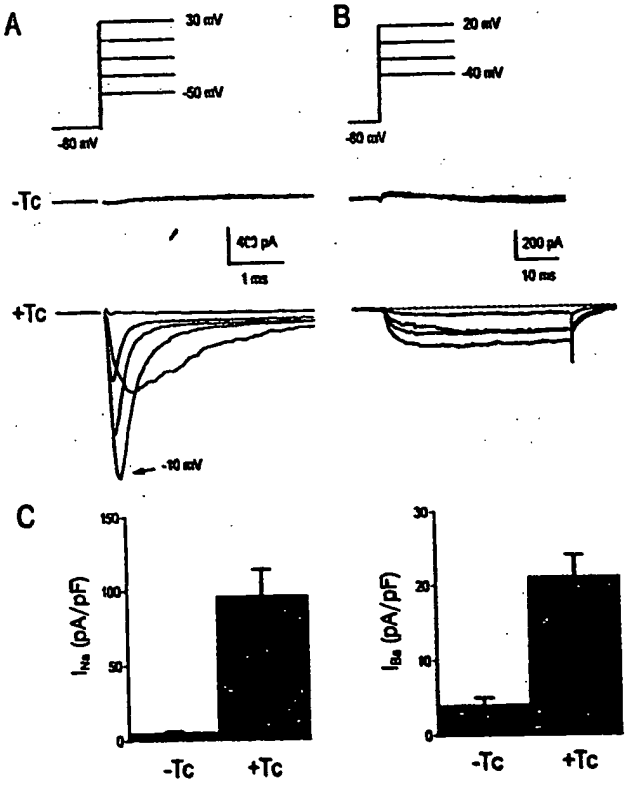

FIG. 5. Cells were grown with FGF-2, without (−Tc) or with (+Tc) tetracycline for 6 days. (*A*) Sodium currents elicited by depolarizations from a holding potential of −80 mV to test potentials of −50, −30, −10, 10, and 30 mV. (*B*) Calcium channel currents elicited by depolarizations from a holding potential of −80 mV to test potentials of −40, −20, 0, and 20 mV. (*C*) Average sodium (I_{Na}) and calcium channel (I_{Ba}) current densities after growth with or without tetracycline. Current densities are current amplitudes normalized to total cell capacitance. Each bar represents the mean from 16 (I_{Na}, +Tc), 12 (I_{Na}, −Tc), 10 (I_{Ba}, +Tc), or 11 (I_{Ba}, −Tc) cells; error bars indicate the SEM.

The differentiation of HC2S2 cells to neurons was demonstrated by morphological and immunocytochemical characteristics f cells. Neurons with phase-bright cell bodies and interconnected long and thin processes expressing specific neuronal markers, tau, NFH, and NeuN were observed. In addition, unlike temperature-sensitive tsA58 immortalized cells, the HC2S2 cells stop expressing nestin upon differentiation (data not shown). Electrophysiological studies have provided further evidence for the differentiation of HC2S2 cells into neurons. Cells treated with tetracycline acquired large sodium currents and the ability to fire action potentials. Moreover, the induced sodium current was of the neuronal form, exhibiting rapid kinetics and a midpoint of inactivation of −60 mV (compared to a midpoint at −80 to −85 mV in glial cells; ref. 30). The restored calcium current was of the high-threshold class, based on the voltage dependence of activation and lack of inactivation (31). Thus differentiated HC2S2 cells exhibit functional properties essential to central nervous system neurons: sodium current, which is necessary for initiation and propagation of the action potential, and calcium current, which is required for neurotransmitter release. Taken together, these results suggest that HC2S2 cells were derived from an immortalized neuronal progenitor cell and that they can be differentiated into neurons after suppression of the v-*myc* oncogene. Moreover, since these cells are derived from the adult hippocampus, progenitor cells with the potential for neural differentiation must exist in the adult nervous system and they are amenable to isolation and expansion *in vitro*. It is now possible to study the mechanism of the cell cycle arrest and subsequent differentiation into neurons that is induced by the down-regulation of the overexpressed v-*myc* oncogene.

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ESTABLISHMENT AND CHARACTERIZATION OF IMMORTALIZED CLONAL CELL LINES FROM FETAL RAT MESENCEPHALIC TISSUE

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Summary

This investigation reports for the first time the establishment of immortalized clones of dopamine-producing nerve cells in culture. Freshly prepared single-cell suspensions from fetal (12-day-old) rat mesencephalic tissue were transfected with plasmid vectors, pSV3neo and pSV5neo, using an electroporation technique. Cells were plated in tissue culture dishes which were precoated with a special substrate and contained modified MCDB-153 growth medium with 10% heat inactivated fetal bovine serum. The immortalized cells were selected by placing the transfected cells in a selection medium (modified MCDB-153 containing 400 μg/ml geneticin). The survivors showed the presence of T-antigens and were non-tumorigenic. Two cell lines, $1RB_3$ derived from cells transfected with pSV_{3-neo} and $2RB_5$ derived from cells transfected with pSV_{5-neo}, revealed only 1 to 2% tyrosine hydroxylase (TH)-positive cells. Repeated single-cell cloning of these cell lines by a standard technique failed to increase the number of TH-positive cells in any clones. Using three cycles of growth, alternating between hormone-supplemented, serum-free medium and serum-containing medium produced a cell line (1RB3A) that was very rich in TH-positive cells. The recloning of 1RB3A yielded clones some of which contained over 95% TH-positive cells. These cells produced homovanillic acid, a metabolite of dopamine, and may be useful not only for neural transplant but also for basic neurobiological studies.

Key words: immortalization; dopamine-producing cells; homovanillic acid; T-antigens; tyrosine hydroxylase.

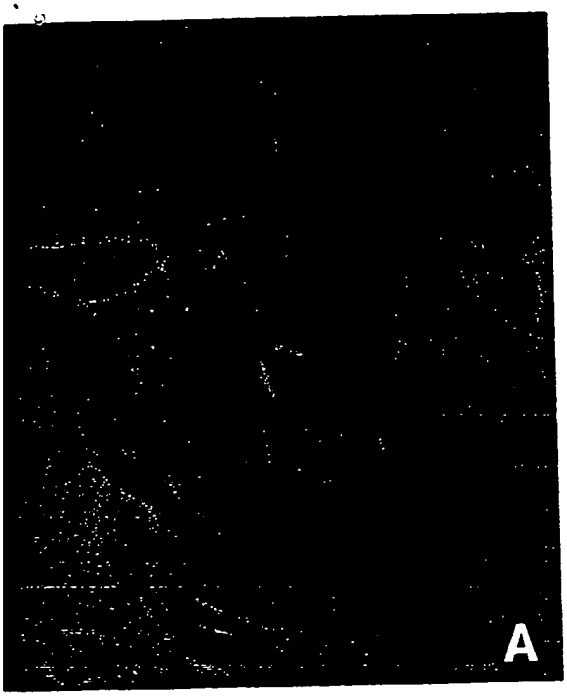
Introduction

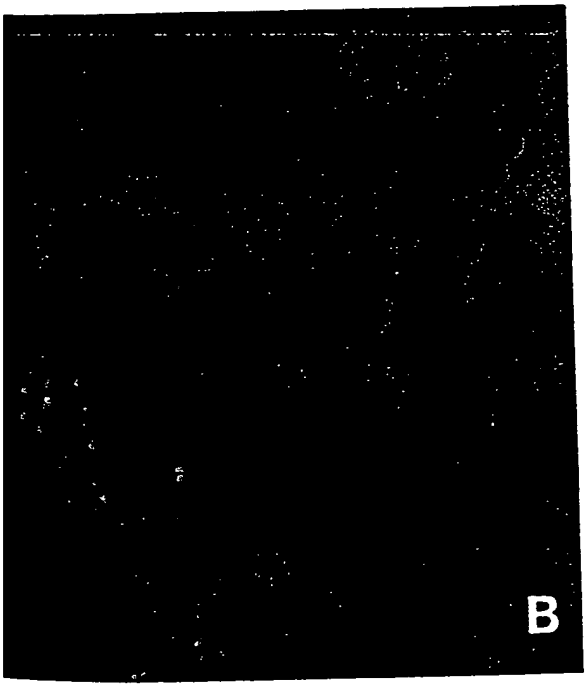
Fetal central nervous system (CNS) and adult peripheral nervous tissues have been transplanted into the brains of animals with specific lesions that are produced by chemical treatments or surgical procedures. These tissue transplants have produced some short-term improvements in behavior deficits of these animals (2,5,6,9,10,14,16,20,36,41–43). Homologous human fetal mesencephalic tissue or autologous adrenal medulla has also been grafted into the brains of several patients with advanced Parkinsonism (1,12,13,17,18,23–26,28–30,38), and some beneficial effects in a few cases have been reported. The use of homologous fetal CNS tissue in neural transplants is limited by ethical issues, limited tissue availability, and some inherent biological problems. The latter includes: a) The presence of donor tissue associated antigen presenting cells that induce rejection of grafted tissues (21); b) poor survival of grafted nerve cells; c) small number of dopamine (DA)-producing cells (about 5%) in tissue; and d) heterogeneity of cell populations. To overcome some of the above difficulties, the establishment of clonal lines of immortalized dopamine-producing nerve cells would be of great value.

The establishment of immortalized nerve cells has been reported by using methods that include somatic cell fusion (8), transfection with retroviral vectors carrying T-antigen genes from SV40 virus (11), or c-*myc* or N-*myc* oncogenes (3,4), and plasmid vectors carrying the SV40-T-antigent gene under the control of a promoter sequence from the gene encoding the rat corticotropin-releasing factor (32). In addition, immortalized brain cells from transgenic mice carrying polyoma virus large T-antigen genes have been established (15). However, they have not been characterized adequately with respect to growth, differentiation, and tumorigenicity. In addition, there is no published study on establishment of immortalized clonal cell lines from rat fetal mesencephalic tissue by plasmid vector, pSV_{3-neo} and pSV_{5-neo}, carrying T-antigen genes from SV40 and polyoma virus, respectively. We now report, for the first time, the establishment of immortalized cell lines ($1RB_3$ and $2RB_5$) from rat fetal mesencephalic cells by transfecting, freshly prepared, single-cell suspensions with plasmid vectors, pSV_{3-neo} or pSV_{5-neo}. These immortalized cells produce T-antigens, exhibit neural-specific proteins, produce dopamine, lack detectable levels of MHC class I and class II antigens, and are non-tumorigenic. Several clones of purified tyrosine hydroxylase (TH)-positive cells which produce dopamine have been isolated from $1RB_3$ cell line.

Materials and Methods

Transfection of freshly prepared cell suspensions with plasmid vectors pSV_{5-neo} or pSV_{3-neo}. The mesencephalic tissues from 12-day-old rat embryos were removed, pooled, and incubated at 37° C in the presence of 0.25% trypsin in phosphate buffered saline (PBS) for 15 min. After incuba





c. 1. Immunofluorescent staining of immortalized fetal mesence-ic cells (2RB5 derived from transfection of cells with pSV_5—) treated a primary antibody to T-antigen showing positive staining (*B*), the same under phase contrast showed neuronal cell morphology (*A*). ×400.

the trypsin solution was removed without centrifugation, and the tis- were thoroughly mixed in modified MCDB-153 growth medium (37) aining 10% heat inactivated fetal bovine serum. The cell suspension centrifuged and the pellet was resuspended in MCDB growth medium. aliquot (0.8 ml) of this was transferred to an electroporation chamber cm), and incubated in the presence of 100 μg of plasmid vectors, pSV_5— or pSV_3— (American Tissue Culture Collection, Rockville, MT. 10 min in ice. The vectors pSV_5— and pSV_3— carry T-antigen gene polyoma and SV40 viruses, respectively, and a neomycin-resistance (39). After incubation in ice, the cells were mixed, electroporated at 25 μF, 0.7 ms, and incubated again in ice for 10 min. The cells placed in 100-mm tissue culture dishes which were precoated with substrate (22). The fresh growth medium was changed after 48 h. cells became confluent they were replated in new precoated dishes the exponential phase of growth were treated with a lethal concentra geneticin (400 μg/ml) for 14 days. Two cell lines ($2RB_5$ derived transfected with pSV_5— and $1RB_3$ derived from cells transfec pSV_3—) were isolated and characterized further with respect to morphology, T-antigen, and cell-specific markers.

Growth media and specialized substrate. Initially, tissue cultur precoated with a special substrate (22) were necessary for the g transfected cells. However, at a later time (about 6 mo. after trans the immortalized cells did not require a special substrate for gro were grown in regular tissue culture dishes. The immortalized cells modified MCDB-153 medium (37) with 10% heat inactivated fet serum for their initial growth. However, after about 1 yr the immo cells started showing slow growth and degeneration in modified M medium. When the cells were shifted to F12 medium contain agammaglobulin newborn bovine serum or RPMI medium with 1 bovine serum, they recovered and grew well. Subsequently, cells faster by about 30% in RPMI than in F12 growth medium. The removed from the dishes by washing them once with 0.25% trypsi (Ca-free minimal essential medium containing 1 m*M* EDTA) and in bating them at 37° C for about 7 min.

Demonstration of T-antigen. Rat immortalized adherent cells in Lab-Tek chamber slides were washed with PBS, air dried at room ature, and fixed in acetone for 5 min. The cells were covered with dilution of a mouse monoclonal antibody (IgG) anti-SV40 T-antigen gene Science, Manhasset, NY) and incubated at room temperature min. The slides were then washed with PBS for 5 min and incubated min with flourescein isothiocyanate (FITC) conjugated goat anti-mo (Capple Lab, CA). The slides were washed again in PBS for 5 mounted with Vectashield mounting medium (Vector Laboratories land Hills, CA). The slides were observed under a fluorescent m with incident light (Zeiss, Germany). Negative controls were prepare ing the same procedure but substituting the primary antibody wi PBS or a 1:50 dilution of normal mouse serum in PBS.

Demonstration of cell-specific markers. Cells from the 2R grown on chamber slides were processed for immunocytochemis the peroxidase anti-peroxidase (PAP) technique or indirect immu scence. Fixation for neuronal markers was in 4% paraform +0.2% gluteraldehyde for 30 min at room temperature, the fix exchanged, and chambers incubated an additional hour at 4 chambers were then washed and incubated for 1 h at room tempera specific antibodies to neurofilament (NF160; 1:1000 dilution hydroxylase (TH; 1:500 dilution) or choline acetyltransferas 3:1000 dilution). One set of slides was also incubated with antibody for the glial marker, glial fibrillary acidic protein (GF Fixation of GFAP was in 100% acetone for 10 min at −2 incubation in primary antibody, cells were washed and incubated ary antibody, either goat anti-rabbit flourescein conjugate (for primary antibodies) or goat anti-mouse rhodamine conjugate (fo nal primary antibodies) for 30 min at room temperature. Chamb processed for PAP according to the method of Sternberger Slides were mounted with 50% glycerol and examined under pha microscopy using our Zeiss microscope's flourescence appa monoclonal anti-NF160 was obtained from Sigma Chemical Co polyclonal anti-TH was a kind gift of Dr. John Reinhard. Wel search Labs, monoclonal anti-ChAT was a kind gift of Dr. F University of Chicago, IL, and polyclonal GFAP was a kind gift o Dahl, Harvard University, Cambridge, MA.

Tumorigenicity of immortalized cells. To test the tumorige mortalized nerve cells, one million cells of $2RB_5$ line were inje neously into each syngeneic Sprague-Dawley rat (a total of 1 athymic mice (a total of 10 animals), and the animals were o period of 60 days after transplantation. $1RB_3A$ line was simila both syngeneic animals and athymic mice.

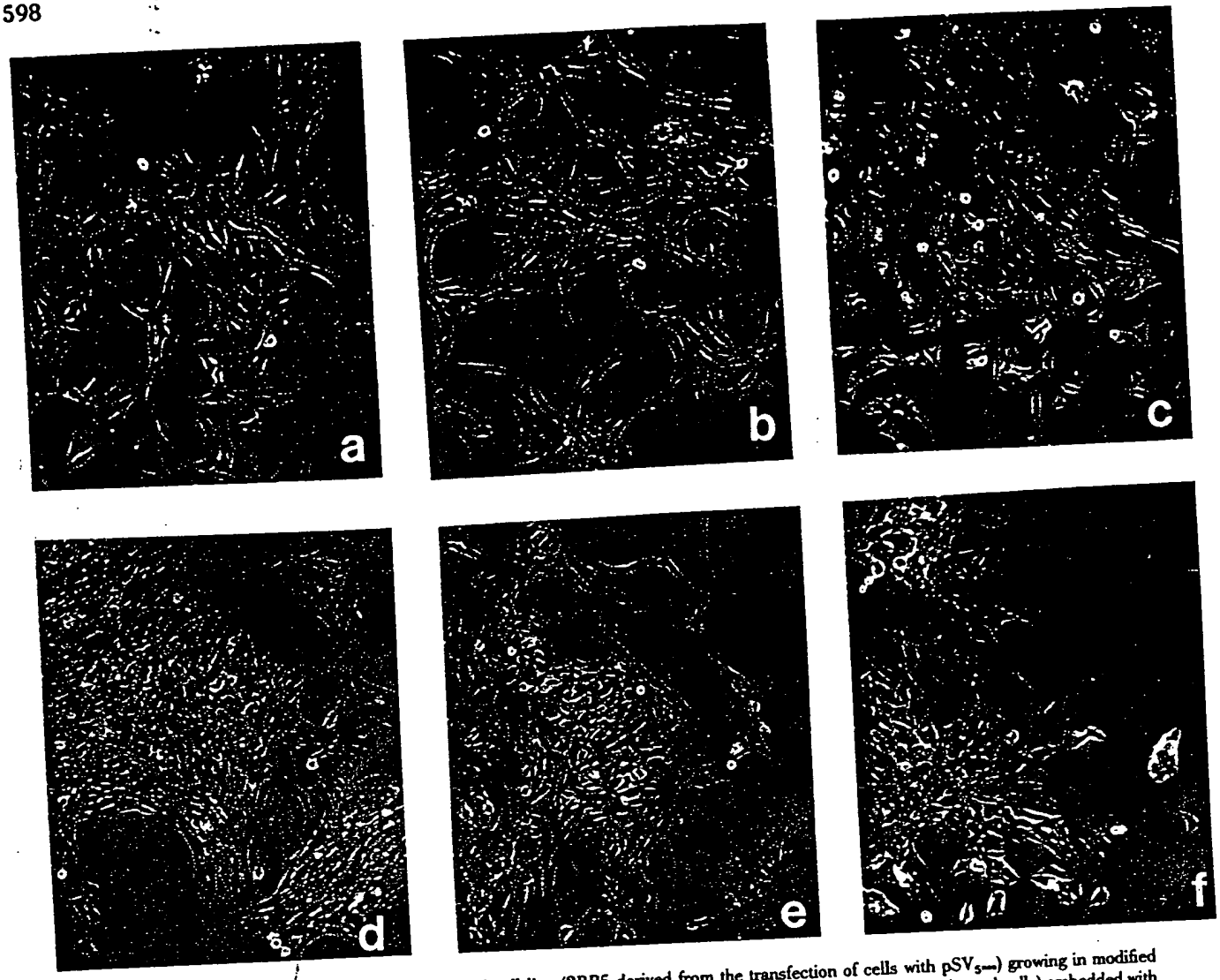


FIG. 2. Photomicrographs of immortalized cell line (2RB5 derived from the transfection of cells with pSV_5—) growing in modified MCDB-153 growth medium. Three cell lines, C2RB5 (*a*), F2RB5 (*b*), and A2RB5 (*c*), showing TH-positive (round cells) embedded with glial cells. Another cell line (1RB3 derived from the transfection of cells with pSV_3—) grown in different growth media, MCDB (*d*), F12 (*e*), RPMI (*f*) showing varied morphology. ×100.

Isolation and characterization of clones. The immortalized cell lines ($1RB_3$ and $2RB_5$) contained only 1 to 2% TH-positive cells. Repeated experiments utilizing a single-cell cloning method, which involves plating of one cell per well in a 100-well plate, yielded several clones (a total of 40 clones). Some clones contained no TH-positive cells, and others continue to show only 1 to 2% TH-positive cells. Due to careful daily examination made under the microscope it was observed that the nerve cells were always embedded in the glial cells; therefore during the plating of one-cell per well, each well actually received two cells, one nerve and one glial. Due to a differential rate of cell division (glial cells divided faster than nerve cells) the resulting clones again contained 1 to 2% TH-positive cells. It has been reported that immortalized precursor CNS cells under appropriate experimental conditions can give rise to either nerve or glia cells (11). The conclusion of this study (11) was that some cells in immortalized cultures may contain multipotent cells which can differentiate into both nerve and glial cells. This may account for the heterogeneity of isolated clones. Another possibility is that the strong attachment between nerve and glial cells never allows formation of clones of pure nerve or glial cells by a standard single-cell cloning method.

To generate clonal cell lines of pure TH-positive cells, an innovative strategy not previously used by any investigator was developed. This strategy involves growing of cells in a modified hormone-supplemented, serum-free F12 medium (SFM) (7) for 4 days followed by serum-supplemented medium for 3 days, and repeating the above procedure 3 times. The addition of SFM caused the death of primarily non-neuronal cells, whereas the presence of serum allowed the growth of both TH-positive and glial cells. The resulting cell line contained 50 to 60% TH-positive cells and produced homovanillic acid, a metabolite of dopamine. This line was recloned and at least 15 clones were tested. Three of them contained over 95% of TH-positive cells. The clones were immunostained with neurofilament-160 and ChAT. The SFM containing insulin (10 μg/ml), progesterone (40 n*M*), transferrin (200 μg/ml), sodium selenite (60 n*M*), and putrescine (200 n*M*) was originally developed by Bottenstein and Sato (7) and later modified by us for the maintenance of differentiated murine NB cells (34). The modification (34) consisted of addition of growth factors at twice the amounts originally proposed.

Assay of TH activity. TH is considered to be a biochemical marker of differentiation for adrenergic nerve cells (33). The activity of TH was measured according to the method of Masserano et al. (31), as modified by Kentroti and Vernadakis (19), and adopted by us (35). The procedure involved the recovery and assay of $^{14}CO_2$ after decarboxylation with partially purified hog aromatic L-amino acid decarboxylase of carboxy-labeled dihydroxyphenylalanine, formed from carboxy-labeled tyrosine. The protein was determined by the method of Lowry et al. (27).

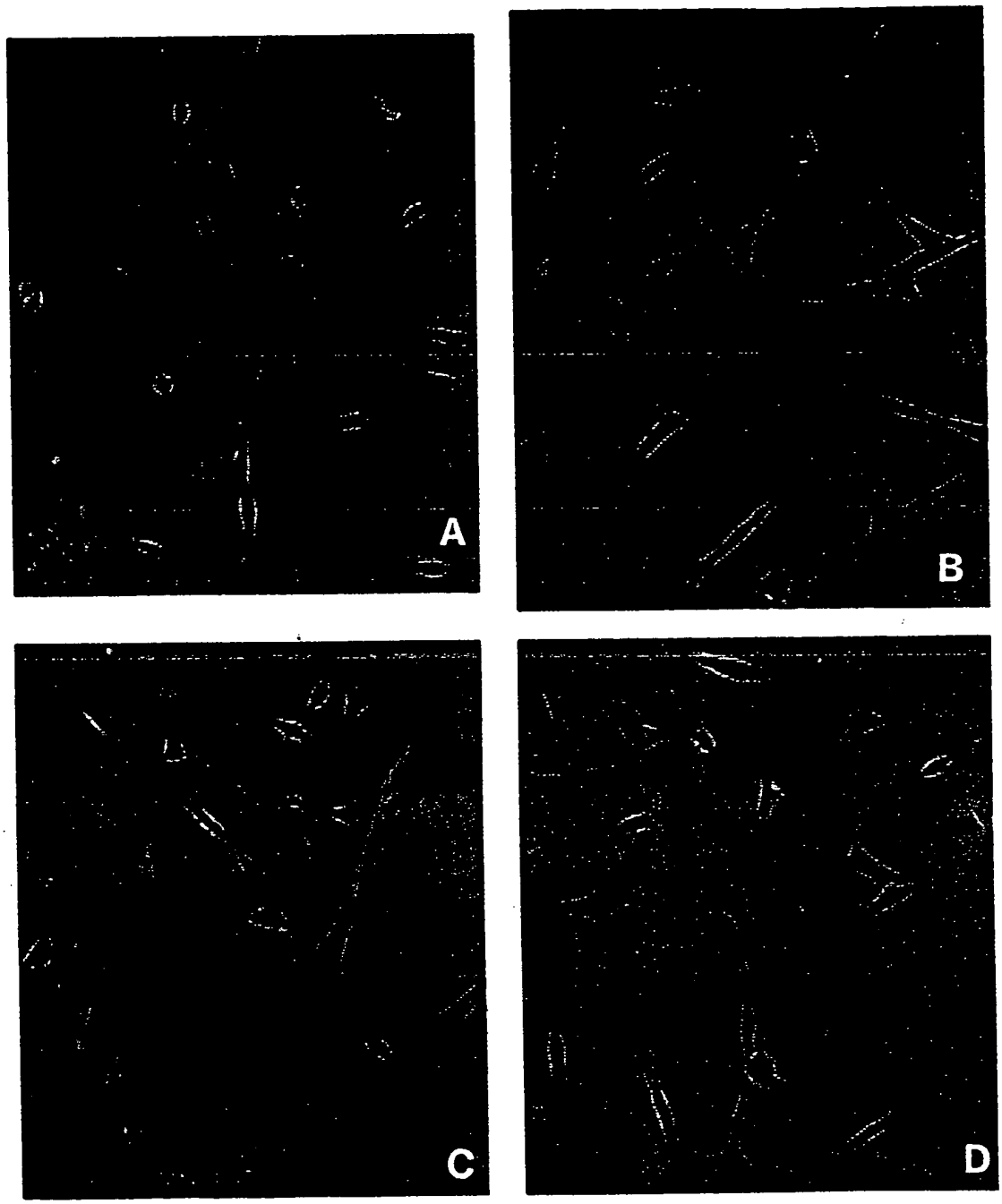


FIG. 3. Cells (2RB5 derived from transfection of cells with pSV_{5-}) stained with primary antibodies to neurofilament-160 (*B*), tyrosine hydroxylase (*C*), and choline acetyltransferase (*D*), using PAP staining. Cells treated without the primary antibody showed no staining (*A*). ×400.

ıssay of homovanillic acid (HVA). Cells ($1RB_3A$) were placed in F12 lium containing 5% human placental serum. The level of HVA, a metab- : of dopamine, was measured 2 days after plating. HVA was assayed n 60 μl of medium following acidification with HCl, extraction into lacetate, and chromatographic separation and measurement by high formance liquid chromatography with electrochemical detection.

Major histocompatability complex (MHC) antigens. Immortalized rat ncephalic cells were cultured in Lab-Tek chamber slides. When the s were confluent, the culture medium was discarded, the chambers ssembled, the slides were washed in PBS and air dried at room tempera- . This was followed by fixation in acetone for 5 min. The chambers were covered with working dilutions of mouse monoclonal antibodies (IgG) specific for class I (6.40.2) and class II (OX6) rat MHC antigens. These antibodies were kindly provided by Don Bellgrau (Barbara Davis Center for Childhood Diabetes, Denver, CO). After 60 min incubation in a humid chamber, the slides were washed twice in PBS for 5 min and then covered with a working dilution of a FITC-conjugated goat IgG (Fab)2 anti-mouse IgG (heavy and light chains) (Cappel Laboratories, catalog no. 11711-3151). After a 30 min incubation, the slides were washed twice and then mounted with Vectashield medium (Vector Laboratories, CA). The slides were observed under a fluorescent microscope with incident light (Zeiss, Germany). Negative controls were prepared following the same procedure

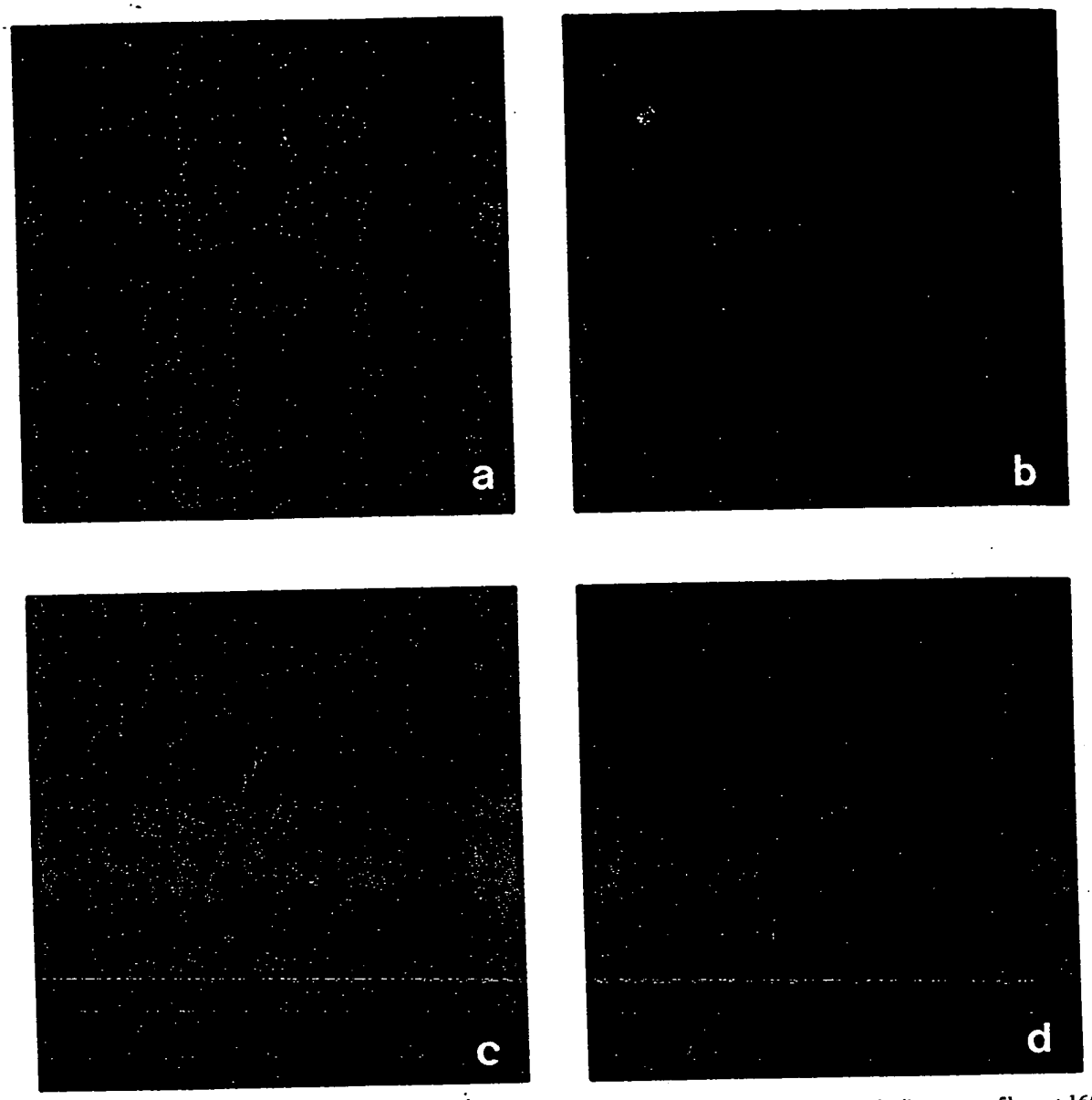


FIG. 4. Immunofluorescent staining (rhodamine) of immortalized cell line ($1RB_3A$) with the primary antibodies to neurofilament-160 (*a*), TH (*b*), and ChAT (*c*), and no primary antibody (*d*). ×400.

but substituting the primary antibodies by PBS or a 1:50 dilution of normal mouse serum in PBS.

RESULTS

Immortalization of cells from fetal rat mesencephalic tissue. Both plasmid vectors, pSV_{5neo} and pSV_{3neo}, were effective in generating immortalized cell lines from fetal rat mesencephalic tissues. The cells transfected with these plasmid vectors grew well in precoated dishes containing modified MCDB-153 medium. When these cells were placed in a selection medium (modified MCDB-153 growth medium containing a lethal concentration of geneticin), extensive cell death occurred. The survivors were presumed to contain T-antigen as well as neomycin resistant genes. Two cell lines, 2RB5 derived from the cells transfected with pSV_{5neo} and 1RB3 derived from the cells transfected with pSV_{3neo} were selected for further characterization.

Presence of T-antigen. More than 95% cells (2RB5) showed positive staining of the nuclei for a T-antigen specific antibody. The staining was characterized by a diffuse granular pattern of the nucleus with a negative staining of the nuclear membrane and the nucleoli (Fig. 1 *B*). The same field under phase contrast showed neuronal cell morphology (Fig. 1 *A*). Similar staining of nuclei was observed in 1RB3 cell line. The cells treated without the primary antibody to T-antigen did not stain (data not shown).

Morphology and growth. Daily observation of the immortalized cells (2RB5) under the phase microscope revealed that cells with different morphology were present in the culture. Three cell lines, C2RB5 (Fig. 2 *a*), F2RB5 (Fig. 2 *b*), and A2RB5 (Fig. 3 *c*) showed

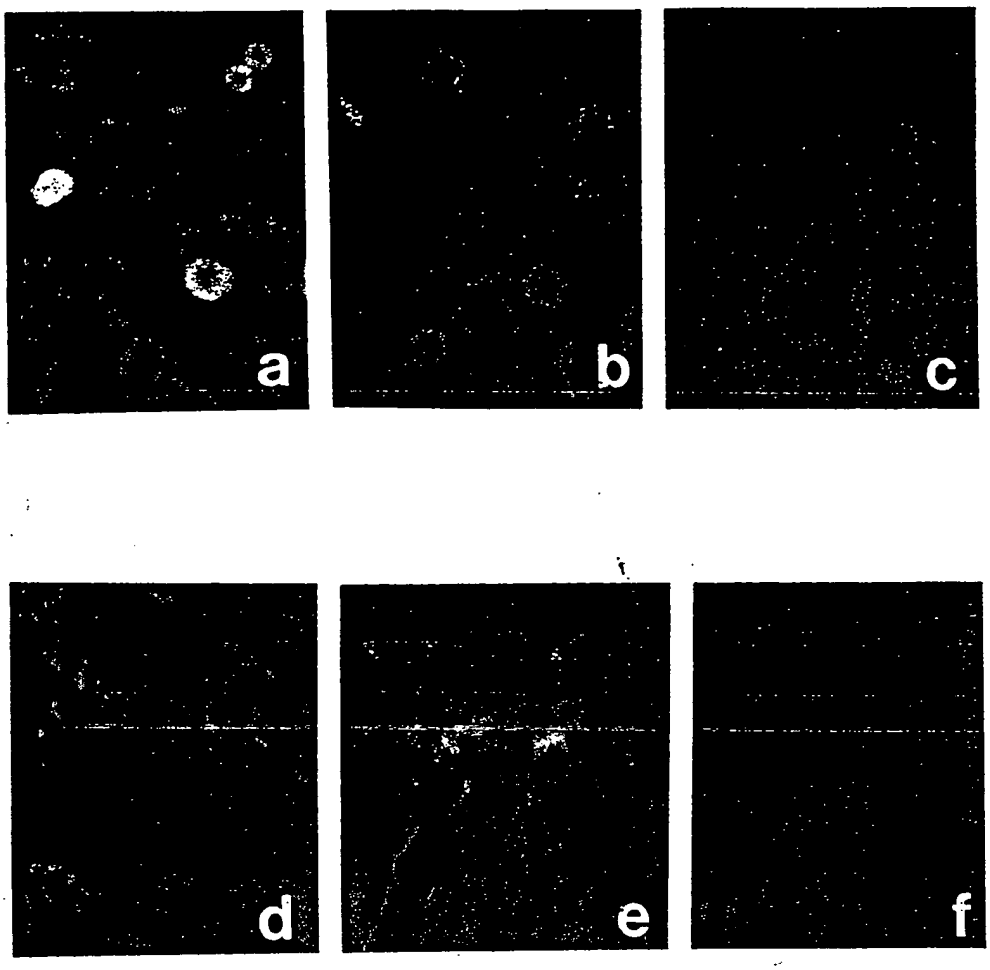


FIG. 5. Immunofluorescent (flourescein) staining immortalized clone ($1RB_3A\text{-}N_{27}$) treated with the primary antibodies to neurofilament-160 (*a*), and TH (*b*), and with no primary antibody (*c*); immortalized clone ($1RB_3A\text{-}N_{32}$) treated with primary antibodies to neurofilament-160 (*d*), and TH (*e*), and with no primary antibody (*f*). *Note* that almost all cells are stained positive for neurofilament-160 and TH. ×400.

· morphology. Most cells were flattened; however, a few cells were embedded in the flattened cells. Initially the trans- cells required a special substrate (25) for their growth, but bout 6 mo. the immortalized cells also grew well in regular culture dishes without any substrate. The immortalized cells ed modified MCDB-153 containing 10% heat inactivated fe- ine serum for their initial growth; however, after about a year owed slower growth and degenerative changes (enlargement s and appearance of one or more intracellular vacuoles). The n of F12 medium with 10% agammaglobulin newborn bo- erum or RPMI medium with 10% fetal bovine serum at this llowed the full recovery of many degenerating cells. Cells in media grew about 30% faster than in F12 medium. There- ll subsequent cell lines were grown in RPMI medium. The ology of cells (1RB3) grown in MCDB (Fig. 2 *d*), F12 (Fig. 2 d RPMI (Fig. 2 *f*) was different.

norigenicity. The immortalized cells when injected subcuta- ly into athymic mice or syngeneic rats did not produce tumors 60 days after transplantation.

sence of neuronal-specific markers. The uncloned immortalized cells (2RB5) stained with the primary antibody to specific neuronal or glial markers. The majority of the cells were stained positively with the antibody to neurofilament-160 (Fig. 3 *B*), a few were stained positively with TH (Fig. 3 *C*), and ChAT stained cells were rare (Fig. 3 *D*). The cells treated without the primary antibody did not stain (Fig. 3 *A*). None of the cells stained with GFAP (data not shown).

Isolation of purified TH-positive clones. The immortalized cell line (1RB3) was selected for cloning. It contained only 1 to 2% TH-positive cells. Repeated (4 times) single-cell cloning did not improve the percentage of TH-positive cells in any clone isolated. This was because TH-positive cells were always firmly embedded with glial cells, and the separation of these cells from each other was not possible during the preparation of single-cell suspension. The addition of SFM caused extensive cell death among glial cells. The treatment of the culture with hormone-supplemental SFM followed by serum-containing medium, and repeating this procedure 3 times, produced cell lines that contained 50 to 60% TH-positive cells. One of them (1RB3A) was stained (rhodamine) for neurofilament-160 (Fig. 4 *a*), TH (Fig. 4 *b*), ChAT (Fig. 4 *c*), and without the

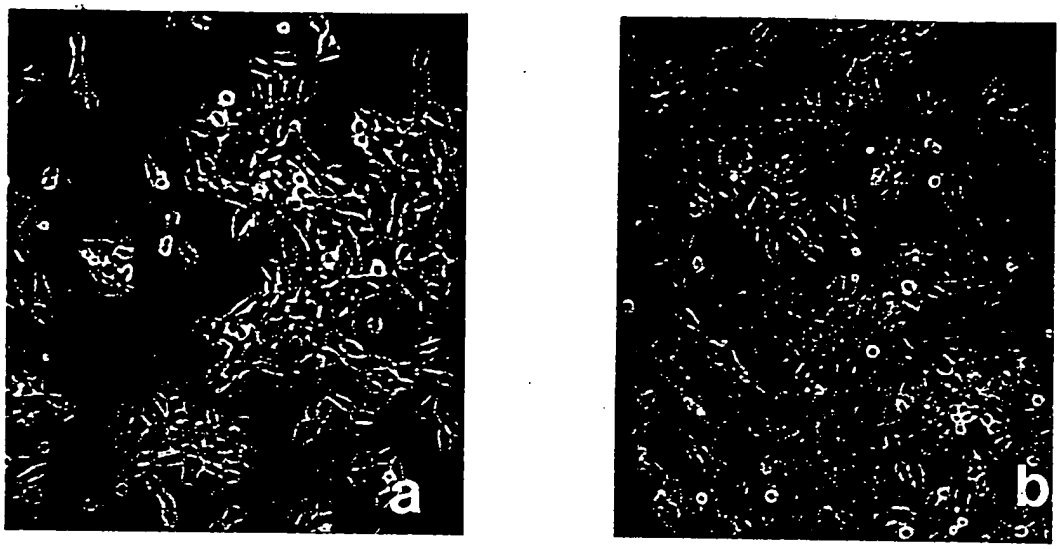


FIG. 6. Photomicrographs of TH-positive clone $1RB_3A\text{-}N_{27}$ (*a*) and $1RB_3A\text{-}N_{32}$ (*b*). These cells are growing in RPMI with a doubling time of about 18 h. ×100.

primary antibody (Fig. 4 *d*). Most of these cells were stained positive for neurofilament and many of them were positive for TH antibody. They also produced homovanillic acid (9.6 ± 2.4 pmole · day^{-1} · well), a metabolite of dopamine. This compared with about 15 pmole · day^{-1} · well of HVA produced by cultured mesencephalic tissue from a 15-day rat embryo which contains about 20 000 dopamine-producing cells. Unlike immortalized cells, these dopamine-producing cells do not divide.

Repeated recloning of the $1RB_3A$ cell line yielded clones some of which contain more than 95% TH-positive cells. Two of the clones (1RB3A-N27 and 1RB3A-N32) were stained (flourescene) with the primary antibodies to neurofilament-160, TH, ChAT, and GFAP. All cells of the 1RB3A-N27 clone were stained positive for neurofilament-160 (Fig. 5 *a*) and TH (Fig. 5 *b*); however, they did not show staining for ChAT or GFAP (data not shown), and they were not stained without the primary antibody (Fig. 5 *b*, *c*). The TH-positive cells were most intense (Fig. 5 *e*) in clone 1RB3A-N32. The neurofilament stain was relatively less intense (Fig. 5 *d*). Two TH-positive clones [1RB3A-N27 (Fig. 6 *a*) and 1RB3A-N32 (Fig. 6 *b*)] were selected for a detailed characterization and for neural transplant studies. Their morphology seemed similar, but 1RB3A-N32 contained cells that exhibited higher levels of TH staining than those in 1RB3A-N27.

Presence of MHC class I and II antigens. No detectable level of MHC class I and II antigens was found in any clone.

DISCUSSION

This is the first report of establishment of immortalized clonal lines of cells from fetal rat mesencephalic tissue. Some of these clones contain over 95% TH-positive cells and produce homovanillic acid, a metabolite of dopamine. Several clones are also TH-negative. Most clones tested show neurofilament-160, but lack ChAT. All immortalized cells produce T-antigens, suggesting that the incorporation of T-antigen genes is primarily responsible for producing immortalized cells. Both plasmids, $pSV_{5-\!-\!-}$ and $pSV_{3-\!-\!-}$, are efficient in producing immortalized cells. These immortalized cells are nontumorigenic when tested in athymic mice or syngeneic rats. Also, there are no detectable levels of MHC class I and II antigens.

The procedures for generating immortalized cell lines by transfecting freshly prepared single-cell suspensions with $pSV_{3-\!-\!-}$ or $pSV_{5-\!-\!-}$ using an electroporation technique are easy to perform, and immortalized cells are obtained within a year. However, the isolation of clones containing primarily TH-positive cells is time-consuming and labor-intensive because the standard single-cell cloning method is inadequate for generating such clones. This is because TH-positive cells are always embedded with glial cells, and a standard method of trypsinization does not separate them. The addition of hormone-supplemented SFM produces extensive cell death among glial cells, whereas serum-containing medium allows the growth of both TH-positive and glial cells. The alternating use of hormone-supplemented SFM and serum-containing media, repeated 3 times, produces cell lines rich in TH-positive cells many of which were no longer embedded with glial cells. The recloning of this cell line ($1RB_3A$) generated clones some of which contain over 95% TH-positive cells. These clones are being transplanted in an animal model of Parkinsonism and can be useful in basic neurobiological studies.

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United States Patent [19]

Boss et al.

[11] **Patent Number: 5,411,883**

[45] **Date of Patent: May 2, 1995**

[54] **PROLIFERATED NEURON PROGENITOR CELL PRODUCT AND PROCESS**

[75] Inventors: **Barbara D. Boss**, Alameda; **Dennis H. Spector**, Oakland, both of Calif.

[73] Assignee: **Somatix Therapy Corporation**, Alameda, Calif.

[21] Appl. No.: **928,676**

[22] Filed: **Aug. 12, 1992**

Related U.S. Application Data

[63] Continuation of Ser. No. 631,617, Dec. 21, 1990, abandoned, which is a continuation-in-part of Ser. No. 456,757, Dec. 26, 1989, abandoned.

[51] **Int. Cl.**[6] **C12N 5/00**

[52] **U.S. Cl.** **435/240.2**; 435/240.1; 435/240.21

[58] **Field of Search** 435/240.1, 240.2, 240.21

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[57] **ABSTRACT**

This invention is based on the development of procedures for isolation and proliferation of neuron progenitor cells and is directed to growth, storage, production and implantation of proliferated neuron progenitor cells. The isolation and culture methods are designed to proliferate mammalian ventral mesencephalon neuron progenitor cells in vitro to produce a culture which differentiates to produce dopamine-producing cells. The products of this invention include a culture containing neuron progenitor cells, preferably, grown as aggregates in suspension cultures. The process of this invention for preparing neuron progenitor cells comprises obtaining ventral mesencephalon tissue from a donor at the appropriate stage of embryonic development; dissociation of the tissue to obtain single cells and small cell clusters for culture; culturing the neuron progenitor cells in an initial culture medium which selects for a novel cell culture containing neuron progenitor cells and growing the cells for a period of time in a second medium, during which the neuron progenitor cells proliferate.

16 Claims, No Drawings

PROLIFERATED NEURON PROGENITOR CELL PRODUCT AND PROCESS

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation of application Ser. No. 07/631,617, filed Dec. 21, 1990 , now abandoned, which is a continuation-in-part of Ser. No. 07/456,767, filed on Dec. 26, 1989, now abandoned.

FIELD OF THE INVENTION

This invention relates to preparation of proliferated neuron progenitor cells and their implantation. In particular, this invention relates to processes for producing proliferated neuron progenitor cells which can be induced in vivo or in vitro to develop into functional neurons which produce dopamine.

BACKGROUND OF THE INVENTION

The nervous system contains two classes of cells: the nerve cells (or neurons) and neuroglia cells (or glia). These cells are distinguished by morphological, biochemical and functional differences. Morphologically, neurons have a cell body and projecting extensions or neurites (processes) of varying length. In vivo, neuritic extensions are further divided into axons (which transfer signals away from the neuron) and dendrites (which transfer signals to the neuron). Among many other biochemical and biophysical processes, neurons synthesize specific chemicals involved in signaling of information. In the central nervous system (CNS) glia are nine times more prevalent than nerve cells. Glia are thought to serve as neural supportive elements by providing nutrients, growth or survival factors and extracellular matrices. These cells are morphologically distinct from nerve cells and do not synthesize neurotransmitters.

To date, attempts to implant functional neuronal cells have largely been unsuccessful. Once the cells send out neurites in vivo, they are very difficult to transplant. The neurites become damaged during preparation of the implantation culture, leading to the death of the cells. In response to this problem, cells for implantation have been obtained from embryos at a point in the embryonic maturation process prior to neurite formation to eliminate cell death due to disruption of the neurites.

However, it is also undesirable to transplant fresh tissue. A period of time to evaluate the tissue prior to implantation, such as to determine whether the tissue is contaminated with a virus, is highly desirable. To hold the tissue for a period of time, the tissue must either be cultured in vitro or frozen. Fresh tissue is fragile, and does not respond well to freezing. The percentage of viable cells in the fresh tissue is greatly diminished by the freeze/thaw process so that a substantial percentage of the cells in the implant are nonviable. In response to the problems inherent in using fresh or fresh-frozen tissue, attempts to culture undifferentiated cells in vitro for implantation have been performed.

Since the initial discovery 80 years ago by Ross Harrison that nerve fibers can survive under tissue culture conditions, the literature has become inundated with reports using cultured nervous tissue. Neuronal cells in vitro also send out neurites, leading to the same problems of cell death upon disruption of the neurites as encountered with fresh tissue. Therefore, attempts to transplant cells prior to production of the neurites in vitro have been made.

The consensus in the reports regarding dissociated CNS tissue (single cell suspension) is that (1) tissue derived from CNS areas which are no longer displaying neuronal division in vivo will only support glial survival in vitro (see, for example, Hansson et al., *Brain Research* 300:9–18 [1984]) and (2) tissue derived from CNS areas still undergoing neuronal division in vivo will allow both neuronal and glial survival in vitro; those neurons will not proliferate, but can differentiate to varying degrees under in vitro conditions (see for example, Ahnert-Hilger et al., *Neuroscience* 17(1):157–165 [1986] and Boss et al., *Dev. Brain Res.*, 36:199–218 [1987]).

Methods which produce cell cultures in vitro in which neuronal cells proliferate are being sought. The method should also minimize the cell loss due to disruption of neurites upon preparation of the cells for implantation. In particular, such cell cultures which would produce dopamine following implantation are highly desirable.

DESCRIPTION OF THE PRIOR ART

A few reports have claimed that a small proportion (6–15%) of neuroblasts obtained from embryonic chick or rat cerebral hemispheres can proliferate under in vitro conditions (see Barakat et al., *Neurochem. Research* 7(3):287–300 [1982]; Kriegstein et al., *Brain Research* 295:184–189 [1984]; Asou et al., *Brain Research* 332:355–357 [1985]; Yoshida et al., *Neurosci. Letters* 70:34–39 [1986]). Temple, *Nature* 340:471–473 (1989) reports that individual CNS blast cells isolated from 13.5 to 14.5 day rat forebrain septal region and cultured as single cells in the presence of conditioning cells can divide and differentiate into neuronal cells, glial cells or both.

Buse et al., *Int. J. Devel. Neurascience* 7:103–113 (1989) report that in cultures of ventricular cells from the rostral part of the mouse neural plates, ventricular cells developing into neuronal phenotypes immediately stopped proliferating upon transfer to cell culture, while a small portion of cells continued to proliferate, displaying morphological characteristics of radial glial cells. The authors conclude that two types of progenitor cells, committed to either neuronal or glial lineages, coexist in cultured neural plate cells.

Others (Honegger et al., *Nature* 282:305–307 [1979]), using reaggregates of dissociated fetal rat brain cells, have demonstrated proliferation (but not specifically neuroblast proliferation) as well as differentiation under the same serum-free conditions. Still other workers (Frederiksen et al., *Soc. Neurosci. Abstr.* 12:1122 [1986]), unable to find significant proliferation in vitro of isolated rat progenitor cells, have turned to genetic manipulation of the cells (using retroviral insertion of oncogenes) to study early developmental events in the CNS.

In a recent article, Wes et al., *J. Cell. Physiol.* 36:367–372 (1988) stated that there is a general consensus that neuroblasts from embryonic rat brains do not divide in culture, despite reports that contradict this belief.

Regarding transplantation of progenitor cells, Doering et al., *Dev. Brain Res.* 5:229–233 (1982) have cultured subventricular epithelial cells from the embryonic mouse neopallium (dorsal cerebral cortex or neocortex) without showing any evidence of proliferation and transplanted these cells into the cerebella of neonatal

mice. The transplanted developing cerebral cortical cells differentiated into their normal phenotypes in vivo, even though misplaced in the cerebellum, but failed to differentiate properly under in vitro conditions.

Cultured embryonic cells have also been used as transplants intended to alter the unusual turning behavior demonstrated by rats injected unilaterally with 6-hydroxydopamine (6-OHDA) (one of the animal models for Parkinson's disease). Brundin et al., *Neurosci. Letters* 16:79–84 (1985) used dissociated rat ventral mesencephalon cells cultured for six days and transported for two days for their striatal transplants. They reported that two out of five transplanted animals showed behavioral recovery, with only 1 in 1000 of the cultured cells surviving as tyrosine-hydroxylase (TH)-positive neurons in the grafts after seven weeks in vivo. No claims were made by the authors that the cells in culture had proliferated to any significant extent.

Kamo et al. *Brain Res.* 397:372–376 (1986), also making no claims regarding proliferation, cultured explants of fetal human paravertebral sympathetic ganglion chains for three weeks to three months prior to using them as aggregates of 1000–4000 neurons for transplantation in their 6-OHDA rat model. Four out of the six transplanted rats showed behavioral recovery as well as surviving TH-positive neurons in the graft (no quantitation reported). Although no immunosuppression was used in these experiments, five out of six grafts, analyzed after 4.5 to 6.0 months, contained surviving TH-positive neurons.

SUMMARY OF THE INVENTION

This invention is based on the development of procedures for isolation and proliferation of neuron progenitor cells and is directed to growth, storage, production and implantation of proliferated neuron progenitor cells. The isolation and culture methods are designed to proliferate neuron progenitor cells in vitro to produce a culture which differentiates to produce dopamine-producing cells. Depending on the culture period and conditions, the progenitor cells differentiate either in vitro or in vivo, following implantation. In addition to increasing the number of neuronal cells, the cultures can be frozen while substantially maintaining cell viability. When cultured as aggregates, the progenitor cell cultures send out neurites which are contained within the aggregates and therefore are not disrupted in the implantation process. Thus, a high percentage of viable cells is maintained.

A portion of the neuron progenitor cells spontaneously differentiate in vitro. Following implantation, a subpopulation of the neuron progenitor cells which did not differentiate in vitro differentiate in vivo and function as tyrosine hydroxylase-containing neurons, gaining the ability to produce functional effects by about three to six months following implantation, depending on the species of donor and host tissue. Alternatively, the neuron progenitor cells can be induced to differentiate in vitro, producing a population of mature neurons which produce dopamine.

The products of this invention include a culture containing neuron progenitor cells. The culture can be progenitor cells or aggregates of progenitor cells in a culture medium, or single or aggregated neuron progenitor cells on or dispersed in a substrate matrix. Most preferably, the cultures are suspension cultures in which the progenitor cells grow as aggregates.

The process of this invention for preparing neuron progenitor cells comprises obtaining ventral mesencephalon tissue from a mammalian donor at the appropriate stage of embryonic development; dissociation of the tissue to obtain single cells and small cell clusters for culture; culturing the neuron progenitor cells in an initial culture medium which selects for a novel cell culture containing neuron progenitor cells (adaptive period) and growing the cells for a period of time (growth period) in a second medium. During the growth period, the neuron progenitor cells proliferate. The progenitor cells differentiate in vivo, following implantation and can be induced to differentiate in vitro by addition of a differentiation agent; e.g., cyclic AMP.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the development of a method for isolating, culturing and proliferating neuron progenitor cells in vitro. The isolation and culture methods are designed to proliferate neuron progenitor cells in vitro to produce a culture having an increased number of neuron progenitor cells. Progenitor cells in the culture can differentiate to produce dopamine-producing cells in vitro, depending on the culture period and conditions, or can differentiate in vivo, following implantation.

In addition to increasing the number of neuronal cells through proliferation, the cultures can be frozen while substantially maintaining cell viability. When grown as aggregates, the progenitor cell cultures send out neurites which are not disrupted in the implantation process, thus maintaining a high percentage of viable cells upon implantation. Following implantation, a subpopulation of progenitor cells which did not differentiate in vitra differentiate in vivo and function as tyrosine hydroxylase-containing neurons, gaining the ability to produce functional effects about three to six months following implantation, depending on the species of the implanted tissue and of the host.

In addition to its use as an implant, a culture of this invention can be used for evaluating agents which inhibit or enhance neuroblast proliferation or differentiation or for evaluating the toxicity of an agent on neurons. Aggregate cultures are particularly advantages for these purposes. Methods for evaluation of pharmacological agents using cell cultures are well known and involve combining the agent with the culture and observing the effect of various concentrations of the agent on the cultured cells.

The process of this invention for preparing neuron progenitor cells comprises obtaining ventral mesencephalon tissue from a mammalian donor at the appropriate stage of embryonic development; dissociating of the tissue to obtain single cells and small cell clusters for culture; culturing the neuron progenitor cells in an initial culture medium which selects for a novel cell culture containing neuron progenitor cells and maintaining the culture for a period of time in a second medium during which the neuron progenitor cells proliferate.

As used herein, the following terms are defined as follows:

"Cell culture" or "tissue culture" refers to the maintenance of cell viability and function in vitra. It may or may not involve cell proliferation.

"Cell proliferation" is a process of cell multiplication by means of cell division. When this occurs in vitro

to any significant level in monolayer cultures, it usually involves one or more subcultures.

"Subculture" means the transfer of cells from one culture vessel to another. The term is synonymous with the term "passage".

"Graft" is a cell culture which has been implanted into a host animal.

Key steps for culturing neuron progenitor cells are described in detail below. While the steps are provided in some detail, it will be readily apparent to a person skilled in the art that the procedures described can be modified and varied without departing from the objective thereof, and this invention is not limited to the specific details presented herein.

PREPARATION OF NEURON PROGENITOR CELL CULTURE

Embryonic mammalian tissue is used to prepare neuron progenitor cell cultures. The tissue can be from any mammalian source, conveniently from a large mammal. Preferably, the tissue is from a goat, cow, sheep or other commercially raised animal. Most preferred is the use of human or porcine tissue.

The donor embryo is in the early stages of development, prior to neurite formation. The tissue is removed from the dopaminergic system of the brain. Preferably, the tissue is from an area which differentiates to form an area of the brain with a relatively high concentration of TH-positive neurons. Most preferably, the tissue is from the ventral mesencephalon.

The objective of the tissue preparation procedure is to disperse the tissue into single cells and small aggregates (about 500 cells per aggregate) without prolonged exposure conditions that impair cell viability. A description of two tissue dissociation methods, mechanical and enzymatic, are described. Mechanical dissociation is preferred since the process is as effective as enzymatic digestion and avoids cell viability impairment caused by exposure to enzymes.

Also described is preparation of monolayer cultures and suspension (aggregate) cultures from the dissociated cells. Aggregate cultures are preferred for implantation, since the procedure allows neuron progenitor cell differentiation within the aggregates during the culture period without disruption of neurites during the implantation process. The aggregate size is controlled by the culture conditions. Preferably, culture conditions are used so that the size of the aggregates remains small enough to implant by injection through a catheter. Aggregate cultures conveniently range in size from about 100 to about 1000 microns.

The neuron progenitor cell culture aggregates have loci of undifferentiated cells and loci of neurons. The loci of undifferentiated cells may contain rosette-like structures in which mitotic figures are often seen. The loci of neurons contain TH-positive cell bodies whose neurites appear to extend to the periphery of the aggregates. Upon histological examination, the aggregates appear to be bordered by a layer of neuropil (an acellular, neurite-rich area).

Preparation of monolayer cultures is also described. Monolayer cultures may be advantageous for procedures in which selection of certain populations of cells is desired. The selected cells can be implanted or cultured as aggregates. Monolayers which will be implanted without intermediate culture as aggregates are preferably grown on a substrate which can be removed from the culture vessel and implanted, such as amniotic membranes, rather than a substrate where enzymatic digestion is required to remove the cell culture for implantation.

Gross examination of typical neuron progenitor cell "monolayer" cultures reveals interconnected three-dimensional structures, rather than the usual two-dimensional monolayer observed with most cell lines. It is thought that the proliferative capacity of these cells creates this three-dimensional effect. Over time, cells begin to migrate from these structures and form typical two-dimensional monolayers in which differentiating neurons and glia can be observed.

If desired, prior to implantation, neuron progenitor cell cultures can be successfully frozen and stored at the time of dissociation or following the completion of the selection period. The cultures can be induced to differentiate in vitro at any time.

In addition to being observably different from prior art neuron cell cultures, the neuron progenitor cell cultures of this invention are biochemically distinguishable from freshly prepared ventral mesencephalon cultures. In particular, for the same amount of embryonic tissue, a neuron progenitor cell culture of this invention produces approximately 100-fold the amount of catecholamines. Following in vitro differentiation, the cultures produce approximately ten-fold the amount of catecholamines of the non-differentiated cultures. In addition, the ratios of concentrations of the three major brain catecholamines (dopamine, epinephrine and norepinephrine) changes following differentiation.

Isolation of Tissue

Removal and Storage of Embryos

1. Remove embryos in amniotic sacs from uterus by sterile lavage with 0.15 M NaCl. Use embryos at Carnegie stages 15–18 for porcine or human tissue. (An explanation of the Carnegie stages can be found in "Developmental Stages in Human Embryos" by Ronan O'Rahilly and Fabiola Muller, Carnegie Institution of Washington (Carnegie Laboratories of Embryology, California Primate Research Center and Departments of Human Anatomy and Neurology; University of California, Davis) Publication 637, 1987 and "An Atlas for Staging Mammalian and Chick Embryos by H. Butler and B. H. Juurlink 1987, CRC Press, Inc.)
2. Store embryos in sterile "Transport Medium" (Jawamoto et al, *Brain Res.*, 384:84–93 [1986]—Formula: 54 mM KH_2PO_4, 30 mM K_2HPO_4, 195 mM D-sorbitol, 5 mM D-glucose, 20 mM sodium lactate, 50 μg/ml gentamicin) at 4° C. for up to 72 hours before proceeding with the cell preparation. That article is incorporated herein by reference in its entirety.
3. All subsequent procedures are performed aseptically.

Dissection of Embryos

1. Remove embryos from amniotic sac.
2. By fine dissection with forceps, peel back the skin from the head of the embryo to expose the meninges and the brain. At this time the fine curvatures of the brain surface and the numerous blood vessels on it will be apparent.
3. Make the first cut, a cross-section through the neural tube, immediately posterior to the isthmus separating the mesencephalon from the metencephalon.

4. Make cuts number 2 and number 3, which separate the dorsal region of the mesencephalon from the ventral region, and discard the dorsal segments.
5. Make cut number 4, at 90° from cut number 1, a cross-section through the neural tube anterior to the mesencephalon, releasing the ventral mesencephalon (VM). Tease away any membranes that are still attached.
6. An ideal VM section should be opaque, white in color, very soft in texture, and shaped roughly like a butterfly. Any trace of clear or semi-clear tissue (i.e. meninges) should be removed from the VM section.
7. Pool dissected VM pieces in a small drop of Hanks Basal Salt Solution (HBSS) (per liter: 0.14 g $CaCl_2$; 0.06 g KH_2PO_4; 0.4 g KCl; 0.1 g $MgCl_2.6H_2O$; 0.1 g $MgSO_4.7H_2O$; 8.00 g NaCl; 0.35 g $NaHCO_3$; 0.09 g $Na_2HPO_4.7H_2O$; 1.0 g D-glucose; 0.01 g phenol red) containing 50 μg/ml gentamicin and 50 mM Hepes buffer, pH 7.2 (Hanks/Hepes/gentamicin, hereinafter HHG). Keep pieces on ice in a small petri dish.

For human tissue, the dissection is essentially the same, but will first require a three-dimensional "reconstruction" of the tissue if it is not intact in order to visualize and identify the region of the mesencephalic flexure.

Following isolation of the tissue, the tissue is dissociated into single cells and small aggregates of cells (less than about 500 cells per aggregate). The dissociation can be by enzymatic or mechanical methods, or a combination thereof. Exemplary enzymatic and mechanical methods are described below. Following dissociation of the cells, the cells are cultured as monolayers or, preferably, in suspension cultures in which the cells form aggregates (aggregate cultures). Each cell culture type is described using a different dissociation procedure. However, any dissociation procedure can be used to prepare monolayer or aggregate cultures.

Aggregate cultures are seeded by placing a small number of cells in a small volume of medium, preferably 1.0 ml or less, more preferably less than 0.5 ml. Not more than about 10^5 cells, preferably fewer than 10^4 cells are seeded per culture. In a most preferred embodiment, from about 2.5×10^3 to about 5×10^3 cells per well are seeded in each well of a 48 well plate. Using seeding conditions that control the initial number of cells per culture tends to control the eventual size of the aggregates. In addition, use of smaller initial seeding concentrations produced smaller aggregates and enhanced the viability of the aggregates. Specifically, the absence of necrotic cells in the centers of the aggregates correlated with lower initial plating densities and maintaining smaller aggregate size.

In the culture process of this invention, the cultures are initially grown in a first culture medium which promotes the survival of neuron progenitor cells which are capable of proliferating in a serum-free, defined medium. The initial culture medium can be a basal medium supplemented with serum, hormones, growth factors and trace elements. Alternatively, the initial culture medium can be a basal medium supplemented with fetal cord serum, preferably from the same species as the cultured cells. Ham's F12 with 5% fetal cord serum is an effective initial culture medium. Preferably, the initial culture medium is Ham's F12 with Chang's supplement C (hereinafter Chang's) or a medium having a similar composition. Changes is preferably present at about 5% (v/v) or more. In a most preferred embodiment, the initial culture medium additionally contains glutamine at a concentration of about 2 mM and superoxide dismutase at about 100 U/ml. The cultures remain in the initial culture medium for at least 4.5 days prior to culture in growth medium. The growth medium is preferably added by day 7, but more preferably between days 4.5 to 5.5.

Mechanical Dissociation of Cells

Preparation of Aggregate Culture

1. The tissue is dissected as described above.
2. Collect tissue sections in a small (about 50 μl) drop of HHG in a small petri dish on ice.
3. Pool all sections in 1 drop of HHG, and chop into pieces 0.5 mm or smaller. Keep this on ice.
4. Remove the cap from a 50 ml centrifuge tube, place a 7×7 cm autoclaved 210 μmesh Nitex screen (Tetko cat. no. 3-210/36) over the opening and secure it in place with an autoclaved rubber band.
5. Repeat step 4 with a second centrifuge tube using a 130 μmesh Nitex screen (Tetko cat. no. 3-130/43).
6. Place 10 ml room temperature HBSS in a 15 ml centrifuge tube that had been rinsed with 4% bovine serum albumin (BSA) in HBSS (hereinafter HBSS/BSA) to form a BSA-coated tube. Add 0.1 ml of DNase stock. (DNase stock is Sigma cat. no. D4527 DNase at 1 mg/ml in HBSS.)
7. Place the tip of a sterile pasteur pipet into the flame of a burner so that melting of the tip closes the opening. Then heat the pipet at about 0.5 cm above the melted tip so that a hook is formed. Coat this pipet by dipping the hooked tip in HBSS/BSA.
8. Using an unaltered, BSA-coated pasteur pipet, transfer the tissue fragments onto the center of the 210 μNitex screen. Wash the tissue with about 2 ml of HHG, then remove as much liquid as possible from the screen with the pipet. Combine this liquid with the HBSS/DNase solution.
9. Using the curve on the hooked Pasteur pipet and a gentle scraping movement, work the tissue through the screen quickly, but gently, to prevent the tissue from drying.
10. Wash the screen with the DNase stock solution. Keep the wetted area to a minimum on the center of the screen.
11. Repeat steps 9 and 10 until no more tissue fragment is visible on the screen, using not more ,than 6 ml of the DNase stock solution.
12. Remove all liquid from the 210μscreen. Then carefully remove the rubber band and the screen.
13. Transfer the cell suspension to the center of the 130 μscreen using the unaltered BSA-coated pipet. Then repeat steps 9 through 12, using the remainder of the DNase stock solution.
14. Transfer the cell suspension from the tube with the 130 μscreen to the BSA-coated 15 ml tube (now empty of DNase stock solution). Then centrifuge at 150×g for 3 minutes.
15. Discard supernatant. Resuspend cells in suitable volume of an appropriate medium, preferably the initial culture medium. In general, 0.5–1.0 ml of F12+5% Chang's supplement C+2 mM glutamine+100 U/ml Superoxide Dismutase is an appropriate volume of a most preferred initial culture

medium. (Chang's supplement C is commercially available from Irvine Scientific; Irvine, Calif.).

16. Remove 10 μl of cell suspension after gentle trituration. Add this to 40 μl of 0.04% Crystal Violet solution, then triturate with a micropipeter until a single nuclear suspension is obtained (about 100×). Count using a hemacytometer.
17. Gently triturate the cells before diluting to appropriate volume for seeding. Usually seeding is done at 3×10^3 cells in 0.3 ml in each well of a 48 well plate.
18. Various volumes of medium can be added 2 hours later to control aggregate formation. In particular, a volume of 0.45 ml yields single aggregates in each well of a 48 well plate. Volumes greater than 0.30 ml but less than 0.35 ml yield multiple aggregates in each well. These volumes were determined at 270 rpm on a mini-orbital shaker (Bellco; Vineland, N.J.). A speed of 180 rpm will yield single aggregates at various volumes. Sylgard 184 (Dow Corning Corp.; Midland, Mich.) coating of the wells may be necessary to inhibit aggregate adhesion to the well (particularly for human tissue).

Switch medium to HN2 Medium 5 days later by removing about half of the medium and adding that volume of HN2 medium. Then feed about twice a week with HN2 Medium by removing approximately half of the medium and replacing that volume with fresh medium. Alternatively, the initial medium can be exchanged for HN2 medium at 5 days and half or all of the medium replaced for subsequent feedings. HN2 medium is a modified version of N2 medium (Bottenstein and Sato, PNAS 76:514–517 [1979]) which contains 1:1 v/v Dulbecco's Modified Eagle Medium (Dulbecco et al., Virology 8:396 [1959]):Ham's F12 (Ham, PNAS 53:288 [1965]). Those articles are incorporated herein by reference in their entirety. The formulae for each of the components of HN2 are shown below. HN2 Medium contains DMEM (low glucose):F12 1:1 v/v and the additional ingredients shown in Table 1. The formulae for DMEM and F12 are found in Tables 2 and 3, respectively.

TABLE 1

HN2 Medium
DMEM (low glucose): F12 1:1 v/v

COMPONENT	CONCENTRATION
N2 medium	
insulin	5 μg/ml
transferrin	100 μg/ml
putrescine	100 μM
selenium	30 nM
progesterone	20 nM
additional components	
glutamine	2 mM
KCl	25 mM
human serum albumin	0.1% (g/100 ml)

TABLE 2

DULBECCO'S MODIFIED EAGLE MEDIUM[1]
Low Glucose

COMPONENT	mg/L
Inorganic salts:	
$CaCl_2$ (anhyd.)	200.00
$Fe(NO_3)_3.9H_2O$	0.10
KCl	400.00
$MgSO_4.7H_2O$	200.00
NaCl	6400.00
$NaHCO_3$	3700.00
$NaH_2PO_4.H_2O$	125.00[2]
Other components:	
D-Glucose	1000.00
Phenol red	15.00
Sodium pyruvate	110.00
Amino Acids:	
L-Arginine HCl	84.00
L-Cystine	48.00
L-Glutamine	584.00
Glycine	30.00
L-Histidine $HCl.H_2O$	42.00
L-Isoleucine	105.00
L-Leucine	105.00
L-Lysine HCl	146.00
L-Methionine	30.00
L-Phenylalanine	66.00
L-Serine	42.00
L-Threonine	95.00
L-Tryptophan	16.00
L-Tyrosine	72.00
L-Valine	94.00
Vitamins:	
D-Ca pantothenate	4.00
Choline chloride	4.00
Folic acid	4.00
i-Inositol	7.20
Nicotinamide	4.00
Pyridoxal HCl	4.00
Riboflavin	0.40
Thiamine HCl	4.00

[1]Dulbecco et al., Virology 8:396 (1959); Smith et al., Virology 12:185–196 (1960); Tissue Culture Standards Committee (TCSC), In Vitro 6(2):93 (1970).
[2]Value shown is in conformance with the TCSC, In Vitro, 9, No. 6 (1970).

TABLE 3

F12 MEDIUM

COMPONENT	mg/L
Inorganic salts:	
$CaCl_2.2H_2O$	44.00
$CuSO_4.5H_2O$	0.00249
$FeSO_4.7H_2O$	0.834
KCl	223.60
$MgCl_2.6H_2O$	122.00
NaCl	7599.00
$NaHCO_3$	1176.00
$Na_2HPO_4.7H_2O$	268.00
$ZnSO_4.7H_2O$[1]	0.863
Other components:	
D-Glucose	1802.00
Hypoxanthine	4.10
Linoleic acid	0.084
Lipoic acid	0.21
Phenol red	1.20
Putrescine 2HCl	0.161
Sodium pyruvate	110.00
Thymidine	0.73
Amino Acids:	
L-Alanine	8.90
L-Arginine HCl	211.00
L-Asparagine.H_2O[1]	15.01
L-Aspartic acid	13.30
L-Cysteine $HCl.H_2O$	35.12
L-Glutamic acid	14.70
L-Glutamine	146.00
Glycine	7.50
L-Histidine $HCl.H_2O$	20.96
L-Isoleucine	3.94
L-Leucine	13.10
L-Lysine HCl	36.50
L-Methionine	4.48
L-Phenylalanine	4.96
L-Proline	34.50
L-Serine	10.50
L-Threonine	11.90
L-Tryptophan	2.04

TABLE 3-continued

F12 MEDIUM COMPONENT	mg/L
L-Tyrosine	5.40
L-Valine	11.70
Vitamins:	
Biotin	0.0073
D-Ca Pantothenate[1]	0.4800
Choline chloride	13.9600
Folic acid	1.300
i-Inositol	18.000
Niacinamide	0.0370
Pyridoxine HCl	0.0620
Riboflavin	0.0380
Thiamine HCl	0.3400
Vitamin B_{12}	1.3600

[1]Value established by the TCSC.

HN2 medium is prepared in polycarbonate tubes and filter sterilized using Milex yellow (i.e. "protein" non-absorbing) filters (Millipore Corp., Bedford, MA). The medium is stored not more than 2 days at 4° C. prior to use.

Enzymatic Dissociation of Cells

Preparation of Monolayer Culture

1. Pool all dissected sections in a drop of HBSS. Chop into pieces not greater than about 0.5 mm with small razor blade holder.
2. Transfer pieces to a 15 ml tube previously rinsed with 4 ml of HBSS/BSA, using a Pasteur pipet previously washed with HBSS/BSA.
3. Centrifuge cells at 75×g for 2 minutes.
4. Resuspend the pellet in a minimum of 0.5 ml Dispase (Collaborative Research cat. no. 40235). This is sufficient volume for less than 5 embryos (as starting material). For 10 embryos, use 1 ml, etc.
5. Incubate 20 minutes at 37° C. with gentle shaking every 5 minutes to resuspend pieces.
6. Add 2 ml HBSS/BSA for every 0.5 ml Dispase.
7. Centrifuge cells at 75×g for 2 minutes.
8. Aspirate supernatant and discard.
9. Resuspend pellet in 0.9 ml HBSS+0.1 ml DNase stock solution. Gently triturate with a BSA-coated, fire-polished Pasteur pipet (bore reduced to about half of its normal diameter) until a homogeneous solution is obtained. Do not leave the cells in the DNase stock solution for more than 5 minutes.
10. Layer cell suspension over 4 ml of HBSS/BSA.
11. Centrifuge at 119×g for 15 minutes.
12. Aspirate the supernatant and discard. Resuspend the cell pellet in culture medium with the BSA-coated, fire-polished Pasteur pipet.
13. Count cells with a hemacytometer.
14. Before proceeding with cells triturate again with BSA-coated Pasteur pipet as cells clump rapidly upon standing.

Seeding Neural Epithelial Cells for Monolayer Cultures

1. Resuspend isolated cells at 5×10^5 cells/ml in F12 medium (Gibco cat. no. 320-1765) containing 5% v/v Chang's Supplement C (Irvine Scientific), and 2 mM glutamine (hereinafter F12+5% Chang's). 2. Seed cells on poly-l-ornithine coated (see below) tissue culture plastic at 0.85×10^5 cells/cm^2. Poly-l-ornithine coated plastic:

   Prepare plastic the day prior to use. Dilute stock poly-l-ornithine (1.0 mg/ml poly-l-ornithine, Sigma P-2533 in sterile 0.2M borate buffer, pH 8) 1:250 v/v in sterile borate buffer. Add an appropriate volume to surface to be coated and incubate 4 hours at 37° C. Transfer plates to 4° C. for overnight storage. Aspirate poly-l-ornithine off the plate and rinse three times with sterile H_2O before use.
3. The day after seeding cells, add an appropriate volume of fresh F12+5% Chang's to the cultures. On day 5 after seeding and every 3 to 4 days thereafter exchange feed, i.e., remove half of the "old" medium and replace with fresh HN2 Medium. When switching the initial culture medium to the growth medium, either all of the medium can be replaced or, preferably, half of the medium can be replaced with new HN2 Medium being used at each subsequent feeding.

Passaging Neural Epithelial Cells

1. Depending on growth, subculture prior to extensive neurite formation.
2. Pool media from wells or plates. Wash each well or plate containing attached cells one time gently with calcium-free, magnesium-free Hanks Basal Salt Solution (CMF-HBSS) and add to above medium.
3. Centrifuge 75×g for 2 minutes at room temperature. Resuspend the pellet in 0.5 ml 0.02% ethylenediamine tetraacetic acid (EDTA), 0.05% trypsin in CMF-HBSS (trypsin-EDTA). Incubate 37° C. for 5 minutes.
4. To washed, attached cells, add an appropriate volume of trypsin-EDTA. When passaging primary and secondary cultures, incubate at 37° C. for 10 to 15 minutes. For later passages, incubate only 5 minutes.
5. Pool trypsinates. Wash wells with F12+5% Chang's and add to trypsinate. Add F12+5% Chang's to equal the volume of the trypsinate.
6. Centrifuge at room temperature at 150×g for 2 minutes.
7. Resuspend the pellet in 0.75 ml HBSS and triturate with fine bore or very fine bore Pasteur pipet just until clumps disappear, usually not more than 20 times.
8. Add 0.75 ml F12+5% Chang's and layer on top of 4 ml of HBSS/BSA.
9. Centrifuge 119×g for 15 minutes.
10. Discard supernatant. Resuspend pellet in 0.75 ml F12+5% Chang's. Triturate ten times or so with very fine bore Pasteur pipet.
11. Count cells in a hemacytometer and resuspend in medium to 5×10^5 cells/ml. Plate on poly-l-ornithine coated plastic at 0.85×10^5 cell/cm^2.
12. Proceed as described for primary monolayer culture above or perform selection procedures, such as FACS (fluorescent-activated cell sorting) or magnetic bead antibody sorting, and reculture in monolayer as described above or reculture in aggregates as described. Following growth of the neuron progenitor cell cultures for 5–15 days, the cultures can be implanted. When convenient, the cultures can be stored frozen prior to implantation. A preferred procedure and thawing cells is described below.

Freezing and Thawing Neural Epithelial Cells The cultures can be frozen and thawed by the following procedure.

Freezing Cells

1. Cells from monolayer cultures are frozen by this procedure following the same procedure used for passaging cells. Aggregates are frozen in same medium, but need not be at any critical concentration of cells. They can be thawed back out as aggregates and recultured or transplanted.
2. Suspend cells in F12+5% Chang's+2 mM glutamine medium (at about 2×10^7/ml for trypsinates of monolayer cultures).
3. Cool on ice for about 5 minutes.
4. Add an equal volume of cold 20% dimethylsulfoxide (DMSO) in F12+5% Chang's dropwise while mixing for a final cell concentration of 10^7/ml.
5. Incubate on ice for 30 minutes.
6. Aliquot into labeled, screw cap freezing vials. Label with cell type, passage number, date and volume frozen.
7. Transfer vials (wrapped in cotton) to a pre-cooled freezing carton at −20° C. for 1 hour.
8. Transfer vials to a −80° C. freezer overnight, then transfer to a liquid N_2-containing dewar flask.

Thawing Cells

1. Remove vials from liquid N_2.
2. Thaw rapidly in 37° C. water bath.
3. As soon as thawed (about 30 seconds for 150 μl), add an appropriate volume of F12+5% Chang's medium at 37° C. to obtain final cell concentration of 2×10^6 cells/ml. At this point cells may be transplanted or passaged further. A preferred transplantation procedure is described below. 4. For passaging monolayer cultures, plate cells on poly-l-ornithine-coated plastic at 3.4×10^5 cells/cm^2.

IN-VITRO DIFFERENTIATION OF PROGENITOR CELLS

Neuron progenitor cells can be induced to differentiate in vitro by adding a differentiation agent to the culture medium at an effective concentration for a time sufficient for progenitor cells in the culture to differentiate. A sufficient period of time can be determined by monitoring the cultures for a significant increase in levels of TH or dopamine.

Differentiation agents include sodium butyrate, butyric acid, cyclic adenosine monophosphate (cAMP) derivatives, phosphodiesterase inhibitors, adenylate cyclase activators and prostaglandins. Effective levels are determined empirically by titration. A preferred differentiation agent is a cAMP derivative. Preferred cAMP derivatives are 8-bromo-cyclic AMP and dibutyryl-cyclic AMP (dbc-AMP). Most preferred is dbc-AMP at a concentration in the range of from about 2 to about 5 mM (final concentration in the culture medium).

The differentiation agent can be added to the culture medium once the cells have been in growth medium for at least about five days. Preferably, the differentiation agent is added prior to ten days in growth medium.

When the growth medium is replaced during the differentiation period, the replacement medium contains the differentiation agent. Differentiation is substantially complete following at least about seven days of continuous exposure to the differentiation agent. Seven days of use of the differentiation agent is optimal. Following completion of differentiation, the differentiation agent is preferably removed. Prolonged exposure to the agent may be toxic. Following completion of differentiation, differetniated progenitor cells in the culture cease proliferation and are preferably transplanted.

When using the differentiated cells for implantation, the time required to cure Parkinsonian symptoms is shorter than when undifferentiated progenitor cell cultures are implanted. Differentiated cells do not require an initial period of time in vivo to differentiate to gain the ability to produce tyrosine hydroxylase.

TRANSPLANTING NEURAL EPITHELIAL CELLS

The neuron progenitor cell cultures can be transplanted by well known procedures for implantation of neural tissue. A preferred procedure is described below. Following transplantation, neuron progenitor cells in the resultant grafts differentiate to produce their differentiated counterparts, neurons. A subpopulation of the neurons are TH-containing neurons which produce functional effects in the host animal.

Preparation of Cells for Transplantation

1. Cells can be transplanted by this procedure:
   a. Immediately after isolation from the embryo;
   b. After passaging;
   c. After growth as an aggregate culture;
   d. After freezing and thawing; or
   e. After selection from a monolayer.
2. Resuspend cells at $1\text{-}5\times10^5$ cells/6 μof HBSS. Hold cells at 4° C. throughout the transplantation procedure.

Transplantation Procedure

1. Weigh and inject the rat with the Ketamine/Xylazine cocktail (Ketamine: Parke-Davis (Vetalar) Morris Plains, N.J.; Xylazine: Rugby (Gemini) Rockville Center, N.Y.) by intraperitoneal (IP) or intramuscular injections to induce surgical anesthesia. IP injections have a quicker onset of action and produce surgical anesthesia for approximately 30–45 minutes.
2. At 5–10 minutes post injection, check the rat for tail reflex. Absence of a tail pinch reflex is a fairly reliable indication that the rat has reached surgical anesthetic plane. Whisker movement and strong corneal reflex are indicators that a supplementary dose should be given (0.05–0.10 ml Ketamine/Xylazine). The cocktail tends to produce a characteristic "bugging" of the eyes.
3. Shave the animal at the site of incision.
4. Due to the length of surgical procedure, rinse the rates eyes with saline and place a drop of mineral oil in its eyes.
5. Position the rat in the ear bars of a Kopf stereotaxic apparatus by inserting one ear bar at a time into the auditory canal. Check the coordinate position of the incisor bar and then position it under the incisor teeth of the rat and pull taut. Close the nose clamp firmly but gently.
6. Make a 0.5" incision from eye midline to just above the ears. Cut the fascia in a crisscross pattern with the surgical knife and scrape the fascia away with a scalpel. Attach the hemostats to the remaining muscle and fascia at the borders of the incision to help reveal the skull surface. 70% alcohol-soaked applicators help to define the sutures. Bregma, lambda and sagittal sutures should be visible if the incision was made correctly.
7. Draw up 6 μl of cell suspension into a 10 μl Hamilton syringe fitted with a 22 gauge, 1" long, blunt-end needle.
8. Mount the syringe in the syringe holder. Align the needle over the bregma suture. Coordinates relate

to anatomical positions within the rat brain, as taken from a stereotaxic atlas. All numbers read on the stereotaxic apparatus. When needle is at bregma (the zero position in the anterior-posterior (AP) and medial-lateral (ML) directions) and skull surface (the Dorsal-Ventral (DV) zero coordinate), these are the zero coordinates. (In calculating the zero coordinate position, it may be necessary to take an average zero coordinate if a poor intersection coronal and sagittal of the sutures exist.) The transplant coordinates are added and/or subtracted from the zero settings. Moving to these coordinates will put the needle into the anatomical area of the brain that corresponds to the transplant coordinates taken from the atlas.

Calculate the surgical structure coordinates desired for the AP, ML positions. Again, lower the needle tip to the surface of the skull but this time at the needle entry site. Record the DV zero coordinates and then calculate the structure depth. Coordinates for transplant: AP+1.0, ML 2.5, DV 6.2 and 4.5 from skull surface, with incisor bar at zero.

9. Mark the location of entry with a ball-point pen. Clear the needle and syringe out of the way by turning the DV knob in a counter clockwise direction and then drill a hole in the skull using a hand-driven pin vise and bit. Be careful to only drill through the skull and not through the dural membrane.
10. Lower the syringe to lowest of DV coordinates.
11. Inject 3 μl of cells at each site, at a rate of 1 μl/min. Wait 2 minutes before raising needle to second site. Wait another 2 minutes before removing needle at a rate of 1 mm/min. Removal rate can be increased to 2 mm/min. for the last couple of millimeters.
12. Remove the rat from the stereotaxic device. Swab any clotted blood, apply wound powder, suture and reapply wound powder on the closed wound (Nitro-fur-wound powder; Life Science Products; St. Joseph, Mo.).

This invention is further illustrated by the following specific, but non-limiting examples. Unless otherwise specified, all temperatures are in degrees centigrade and all percentages are in weight percents. Procedures which have been reduced to practice are presented in the past tense, and procedures and products which are first reduced to practice in the filing of this application are presented in the present tense.

EXAMPLE 1

Fixation of Brain

Rat brain tissue was fixed by the following procedure.

1. The rat is deeply anesthetized with pentobarbital.
2. Set the pump on 45 (about 30 ml/min) and prime the tube with 0.1M phosphate buffered saline (hereinafter PBS or 0.1M PBS) (Formula: 14.8 g NaH_2PO_4, 4.3 g Na_2HPO_4, 7.2 g NaCl for 1 L. of 0.1M PBS).
3. Open the thoracic cavity, clamp off the descending aorta and insert the needle into the left ventricle. Cut a small hole in the right atrium and start the pump.
4. Perfuse 100–200 ml of PBS, turn off the pump, transfer the tube to the 4% paraformaldehyde without creating air bubbles, and restart the pump. Perfuse approximately 250 ml. Turn the pump off, disengage the needle, rapidly decapitate the rat, and dissect out the brain.
5. Place the brain in 4% paraformaldehyde (4% paraformaldehyde in 0.1M PBS) for 2 hours. Wash in PBS and transfer to 30% sucrose with 0.1% sodium azide overnight and hold no longer than 1 week for sectioning.

EXAMPLE 2

Sectioning of Fixed Brain Tissue

Brain tissue which was fixed according to the procedure in Example 1 was sectioned by the following procedure.

1. Cut into blocks about 7 mm thick.
2. Snap freeze blocks in dry ice/ethanol.
3. Cut frozen blocks on a sliding microtome at 40μ.
4. Store sections at 4° C. in 0.1M PBS with 0.1% Na azide up to 2 weeks before staining for tyrosine hydroxylase (TH). For long term storage: 30% ethylene glycol, 25% glycerin in 0.05M phosphate buffer.

EXAMPLE 3

Tyrosine Hydroxylase Staining of Sections

Sections prepared according to the procedure described in Example 2 were stained for tyrosine hydroxylase by the procedure described below.

Day 1

1. Wash sections in 0.1M PBS pH 7.4, two times, 5 minutes each.
2. Incubate in 3% hydrogen peroxide in distilled water for 5 minutes to block endogenous peroxidase activity.
3. Wash briefly in distilled water.
4. Wash in PBS three times, 5 minutes each.
5. Incubate in PBS with 3% goat serum (normal goat serum, Vector Labs; Burlingame, Calif.) for 30 minutes (to block non-specific staining when using a primary rabbit antibody and a secondary goat anti-rabbit antibody).
6. Wash in PBS three times, 5 minutes each.
7. Incubate in tyrosine hydroxylase antibody (Eugene Tech International; Allendale, N.J. at 1:500 dilution or East Acres; Southbridge, Mass. at 1:2000 dilution for rat brains and 1:2500 dilution for mouse brains) diluted in PBS with 1% goat serum and 0.1% Triton X-100 overnight at room temperature, agitated on a fish-tank aerator.

Day 2

8. Wash in PBS with 1% goat serum three times, 5 minutes each.
9. Incubate in Biotinylated anti-Rabbit IgG (Vector, Burlingame, Calif. reconstituted in 1 ml, use 1:300 dilution in PBS) for 1 hour.
10. Wash in PBS with 1% goat serum three times, 5 minutes each.
11. Incubate in ABC solution (Vector, Burlingame, Calif. dilute each solution A and B 1:100 in PBS, then let sit for 30 minutes before incubation) for 1 hour.
12. Wash in PBS three times, 5 minutes each.
13. Incubate in 3,3 diaminobenzidene (DAB) substrate (Litton Bionetics, Durham, N.C.; reconstitute in 1 ml 0.01M phosphate buffer (PB) [use 0.1M phosphate buffer diluted 1:10], then dilute DAB

1:10 with 0.01M PB, filter and add 2 μl/ml of 30% Hydrogen Peroxide) for 10 minutes.
14. Wash in tap water.
15. Mount the floating sections from PBS on subbed slides, (slides dipped in 0.25% chromium potassium sulfate and 2.5% gelatin in distilled water, then dried) dry, wash in distilled water, dehydrate in 95% EtOH 2 minutes, 100% EtOH two times, 2 minutes each, then Hemo-De (Xylene substitute, Fisher Corporation, Springfield, N.J.) three times, 2 minutes each, and coverslip using Protexx mounting media (VWR, San Francisco, Calif.).

EXAMPLE 4

Quantitative Analysis of TH-Positive Cell Bodies in Sections from Grafted Brains

Tyrosine hydroxylase (TH) positive staining was used as a marker for those neurons which contain dopamine. TH-positive cell bodies in the graft area which had been stained by the procedure described in Example 3 were quantitated by the procedure described below.

1. All TH-positive cell bodies are counted in every third section throughout the graft using a Zeiss microscope at 100 × magnification.
2. The section thickness is measured at 1000× using an oil immersion objective. Thickness in microns is found by focusing on the top of the section, recording the micrometer reading on the focusing knob of the microscope, and then focusing through the section to the bottom and computing the difference in readings. The thickness is measured in two different areas on five representative sections.
3. The cell body length is measured at 1000 × magnification using an ocular micrometer or the video image analyzer. Ten typical cell body lengths are measured per section on ten representative sections. Due to shrinkage in the section thickness and cell diameter during histological preparation, these measurements must be taken at the time of counting.
4. Approximation of the number of cells in the sections not counted is done using the formula being:

$$A_1=A+[(B-A)/3] \text{ and } A_2=A+2[(B-A)/3]$$

where: A=the number of cells counted in the first section, and B=the number of cells counted in the second section, and A_1 and A_2 are the number of cells from the sections not counted between A and B.

5. Abercrombie correction for double counting cell in adjacent sections was used to correct the approximation. The formula is:
corrected number=(experimental cell number). [(average section thickness) / (average cell diameter +average section thickness)]

EXAMPLE 5

Implantation of Progenitor Cell Cultures In Vivo

Grafts of cultured fetal pig progenitor cell cultures of this invention function in the Parkinsonian rat model. (The Parkinsonian rat model was described by Strecker et al., *Exp. Brain Res.* 76:315–322 [1989]and Brundin et al., *Neurosci. Lett.* 61:79–84 [1985]. Those articles are incorporated herein by reference in their entirety.) Donor tissue was dissected from the ventral mesencephalon of stage 15–18 fetal pigs (21–24 day fetal pigs), enzymatically dissociated, mechanically triturated, and cultured as described above for aggregate cultures for 15 days. Typically, 3×10^4 cells were plated per well; and, after 6–150× proliferation, the cells were transplanted (1 well/rat). The cultured tissue was injected directly into the dopamine-denervated striata of host rats. By 16 weeks post-implantation, eleven of 20 grafted rats have shown behavioral recovery in the amphetamine-induced rotation test. Histological analysis revealed very large grafts containing numerous dopamine neurons as identified by tyrosine hydroxylase (TH) immunohistochemistry according to the procedure described in Examples 1–4.

The average density of the TH-positive neurons in functioning grafts was found to be greater than 100 cells/mm^3. Most commonly, TH-positive neurons were found to be situated at the graft periphery. Occasionally, rosette-like structures were observed within the grafts.

A parallel experiment combining immunohistochemistry and autoradiography has shown that when such cultures are labeled for 2 days in vitro with $_3$H-TdR (days 5–7) and grafted at day 7, numerous TH-positive grafted neurons containing label are found at 4 weeks post-grafting. That experiment is described in Example 6.

EXAMPLE 6

Proliferation of Progenitor Cell Cultures In Vitro

1. Donor tissue was dissected from the ventral mesencephalon of stage 16 fetal pigs, enzymatically dissociated, and cultured as described for aggregate cultures for 7 days.
2. From day 5 to day 7, the cells were labelled with tritiated thymidine, at 0.1 μCi/ml (New England Nuclear, 20 Ci/mM, 1 mCi/ml, Boston, Mass.)
3. On day 7, medium was removed, cells were washed three times with HBSS, and transplanted as described into nude mice.
4. Brains were sectioned as described after 4-and 8-week transplant times.
5. Sections were stained for TH as described.
6. Slides were prepared for autoradiography: slides were dipped into NTB2 (1:1 with H_2O) (all reagents: Kodak, Rochester, N.Y.), and air dried in the dark.
7. Slides were exposed at 4° C. for 2 weeks.
8. Slides were warmed to room temperature, and developed in 50% Dektol for 1 minute.
9. Slides were rinsed in H_2O for 10 seconds.
10. Slides were fixed in 25% Kodafix for 4 minutes.
11. Slides were rinsed in H_2O for 10 minutes before drying and coverslipping.
12. TH(+) cells were counted, and the percentage of those cells which had incorporated label into their nuclei was determined.
13. 50% of all TH(+) cells had incorporated thymidine between days 5 and 7 in culture.

EXAMPLE 7

Minimum TH(+) Cell Density Required For Cure

1. Twenty-seven ablated rats were transplanted with stage 15–18 porcine tissue (day 21 to 24), either fresh or aggregated in culture, by techniques described.

2. The rats were analyzed for function by the procedure described in Strecker et al., Exp. Brain Res. 76:315–322 (1989) during a 16-week period post-implantation.
3. The total number of TH(+) cells in each graft was counted as described.
4. The area of the graft in each section was calculated with the use of Vidometric 150 software (American Innovision, San Diego, Calif.).
5. The total volume of each graft was calculated using the same formula used for determining the total number of TH(+) cells in the graft, as described.
6. The number of TH(+) cells/mm^3 was determined for each graft.
7. The results indicated that for 100% functional recovery at least 100 TH(+) cells must be present in each mm^3 of tissue.
8. Comparison of the group of transplants which showed 100% functional recovery to the group of transplants which showed either partial or no functional recovery by a Mann-Whitney test showed that the densities of the 2 groups are significantly different ($p<0.0005$).

EXAMPLE 8

In Vitro Induction of Differentiation

Eight replicate aggregate cultures of human neuron progenitor cells prepared as described above were switched from initial medium to growth medium on day 5. On day 14, the following concentrations of potential differentiation agents, retinoic acid and di-butyryl cyclic AMP (dbc-AMP) were added to the cultures.

Retinoic acid ($\times 10^{-7}$M): 0; 0.2; 1.0; 5.0

dbc-AMP (mM): 0; 0.4; 2.0; 10.0

Following seven days in the presence of the differentiation agent, the cultures were evaluated for the amount of tyrosine hydroxylase (TH) DNA by standard protein immuno-slot-blot techniques. The cultures without differentiation agent were used as controls. All of the cultures with added retinoic acid had from about 80 to 110% of the amount of TH as the control cultures. For the cultures with dbc-AMP, the amount of TH production was significantly increased as shown below.

dbc-AMP (mM)	0.4	2.0	10.0
% of control DNA	400	650	175

The study demonstrates that the amount of TH DNA in the cultures was significantly increased using dbc-AMP, but was unchanged using retinoic acid.

Six replicate samples of two porcine cultures from two different litters (designated 1133–44 and 1016–94) were switched to growth medium at day 5 and grown for 10 days in the absence of a differentiation agent followed by 7 days in the presence of the concentrations of dbc-AMP stated below. The amount of TH/DNA was evaluated by slot-blot as described above. The results are illustrated below.

	% of Control Culture	
dbc-AMP (mM)	1133-44	1016-94
0	100	100
0.5	125	140
1.0	130	160
2.0	175	260
5.0	275	330
10.0	200	200

As shown in the table, the most effective concentration range for dbc-AMP as a differentiation agent is from 2.0 to 5.0 mM.

EXAMPLE 9

Analysis of Catecholamines of Neuron Progenitor Cell Cultures

The catecholamine content of neuron progenitor cell cultures was studied to characterize changes in catecholamine production following in vitro induction of differentiation. An HPLC analysis determined the approximate concentration and ratios of the three major brain catecholamines; dopamine, epinephrine and norepinephrine.

The cell cultures analyzed included a "control" culture. The control culture was freshly isolated cells from ten porcine embryos (21-day ventral mesencephalon) prepared as described in Example 5. The "standard" culture was a neuron progenitor cell culture of this invention prepared as described in Example 5 from the same embryo preparation as the control culture and cultured for two weeks. That culture included approximately one embryo-equivalent of cells. The third culture was a two week progenitor cell culture prepared as the standard culture and then differentiated using 5 mM dibutyryl cyclic AMP for one additional week as described in Example 8 (for a total of three weeks in culture). That culture included approximately one-third embryo-equivalents of cells.

The HPLC analysis determined that the freshly prepared culture did not have detectable amounts of catecholamines. (The HPLC method can detect the presence of 10 picograms of catecholamines.) The standard culture had at least about 100 picograms of catecholamines (at least 100× the catecholamine content per embryo equivalent of freshly isolated cells). The differentiated culture had several fold greater catecholamine content (at least 3×) than the standard culture (at least 10× the catecholamine content per embryo equivalent of the standard culture).

In addition to producing substantially more catecholamines than the standard culture, the differentiated neuron progenitor cell culture produced different proportions of the three major brain catecholamines. The ratio of the concentration of dopamine to epinephrine to norepinephrine for the standard culture and the differentiated culture are shown below.

	dopamine	epinephrine	norepinephrine
standard	1.00	0.85	0.49
differentiated	1.00	0.99	0.63

This Example demonstrates that the cultures of this invention are biochemically distinguishable from freshly isolated ventral mesencephalon cells from which they were derived. The Example also demon-

strates that in addition to the observable differences in the cell cultures following differentiation which are described in Example 8, the cultures also produce more catecholamines having a different ratio of the major brain catecholamines.

What is claimed is:

1. A culture consisting essentially of mammalian ventral mesencephalon neuron progenitor cells or mammalian ventral mesencephalon neuron progenitor cells and their differentiated counterparts, said neuron progenitor cells being taken from a region of the brain that produces dopaminergic cells at a stage of development when the region of the brain has no dopamine-containing cells, said neuron progenitor cells in said culture having undergone at least one round of cell division after dissociation of tissue used to establish said culture, said progenitor cells in said culture being capable of seven to eight rounds of cell division after dissociation of said tissue.

2. The culture of claim 1 wherein said progenitor cells are porcine.

3. The culture of claim 1 wherein said progenitor cells are human.

4. The culture of claim 1 wherein said culture is capable of curing Parkinsonian symptoms about 4 months following implantation.

5. The culture of claim 1 wherein said culture comprises cell aggregates.

6. The culture of claim 5 wherein said aggregates range in size from about 100 to about 1000 microns.

7. The culture of claim 5 wherein said aggregates have loci of undifferentiated cells and loci of neurons.

8. A method for evaluating an agent which inhibits or enhances neuroblast proliferation or differentiation or for evaluating the toxicity of an agent on neurons comprising combining said agent with a culture of claim 1.

9. A method for producing a culture comprising neuron progenitor cells comprising:

a. obtaining ventral mesencephalon tissue from a mammalian embryo at Carnegie stages from 15 through 18;

b. dissociation of said tissue to obtain single cells and small cell clusters for culture;

c. culturing said single cells and small cell clusters in an initial culture medium which promotes the survival of neuron progenitor cells which are capable of proliferating in serum-free, defined medium for a sufficient period of time to produce a cell culture containing neuron progenitor cells capable of proliferating in serum-free, defined medium; and

d. growing said cell culture containing neuron progenitor cells in a medium which maintains neuronal cells.

10. The method of claim 9 wherein said medium of step (c) is Ham's F12 medium supplemented with Chang's supplement C.

11. The method of claim 10 wherein said medium is additionally supplemented with glutamine and superoxide dismutase.

12. The method of claim 9 wherein said medium of step (d) is HN2 medium.

13. The method of claim 16 wherein said medium of step (d) is HN2 medium.

14. The method of claim 9 wherein said period of time is 5 days.

15. The method of claim 9 wherein said culture is an aggregate culture.

16. The method of claim 14 wherein not more than 10^5 cells are seeded per culture.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,411,883
DATED : May 2, 1995
INVENTORS : Barbara D. Boss and Dennis H. Spector

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 22, line 27, delete "HN2" and insert --N2--

Column 22, line 28, delete "16" and insert --12--

Signed and Sealed this

Twelfth Day of December, 1995

Attest:

Attesting Officer

BRUCE LEHMAN

Commissioner of Patents and Trademarks

US005750376A

United States Patent [19]

Weiss et al.

[11] Patent Number: 5,750,376

[45] Date of Patent: May 12, 1998

[54] **IN VITRO GROWTH AND PROLIFERATION OF GENETICALLY MODIFIED MULTIPOTENT NEURAL STEM CELLS AND THEIR PROGENY**

[75] Inventors: **Samuel Weiss**; **Brent Reynolds**, both of Alberta, Canada; **Joseph P. Hammang**; **E. Edward Baetge**, both of Barrington, R.I.

[73] Assignee: **NeuroSpheres Holdings Ltd.**, Calgary, Canada

[21] Appl. No.: **483,122**

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Related U.S. Application Data

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[52] **U.S. Cl.** **435/69.52**; 435/69.1; 435/172.3; 435/325; 435/368; 435/377; 435/384; 435/392; 435/395

[58] **Field of Search** 435/240.2, 172.3, 435/69.1, 69.52, 325, 368, 377, 384, 392, 395

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[57] **ABSTRACT**

A method for producing genetically modified neural cells comprises culturing cells derived from embryonic, juvenile, or adult mammalian neural tissue with one or more growth factors that induce multipotent neural stem cells to proliferate and produce multipotent neural stem cell progeny which include more daughter multipotent neural stem cells and undifferentiated progeny that are capable of differentiating into neurons, astrocytes, and oligodendrocytes. The proliferating neural cells can be transfected with exogenous DNA to produce genetically modified neural stem cell progeny. The genetic modification can be for the production of biologically useful proteins such as growth factor products, growth factor receptors, neurotransmitters, neurotransmitter receptors, neuropeptides and neurotransmitter synthesizing genes. The multipotent neural stem cell progeny can be continuously passaged and proliferation reinitiated in the presence of growth factors to result in an unlimited supply of neural cells for transplantation and other purposes. Culture conditions can be provided that induce the genetically modified multipotent neural stem cell progeny to differentiate into neurons, astrocytes, and oligodendrocytes in vitro.

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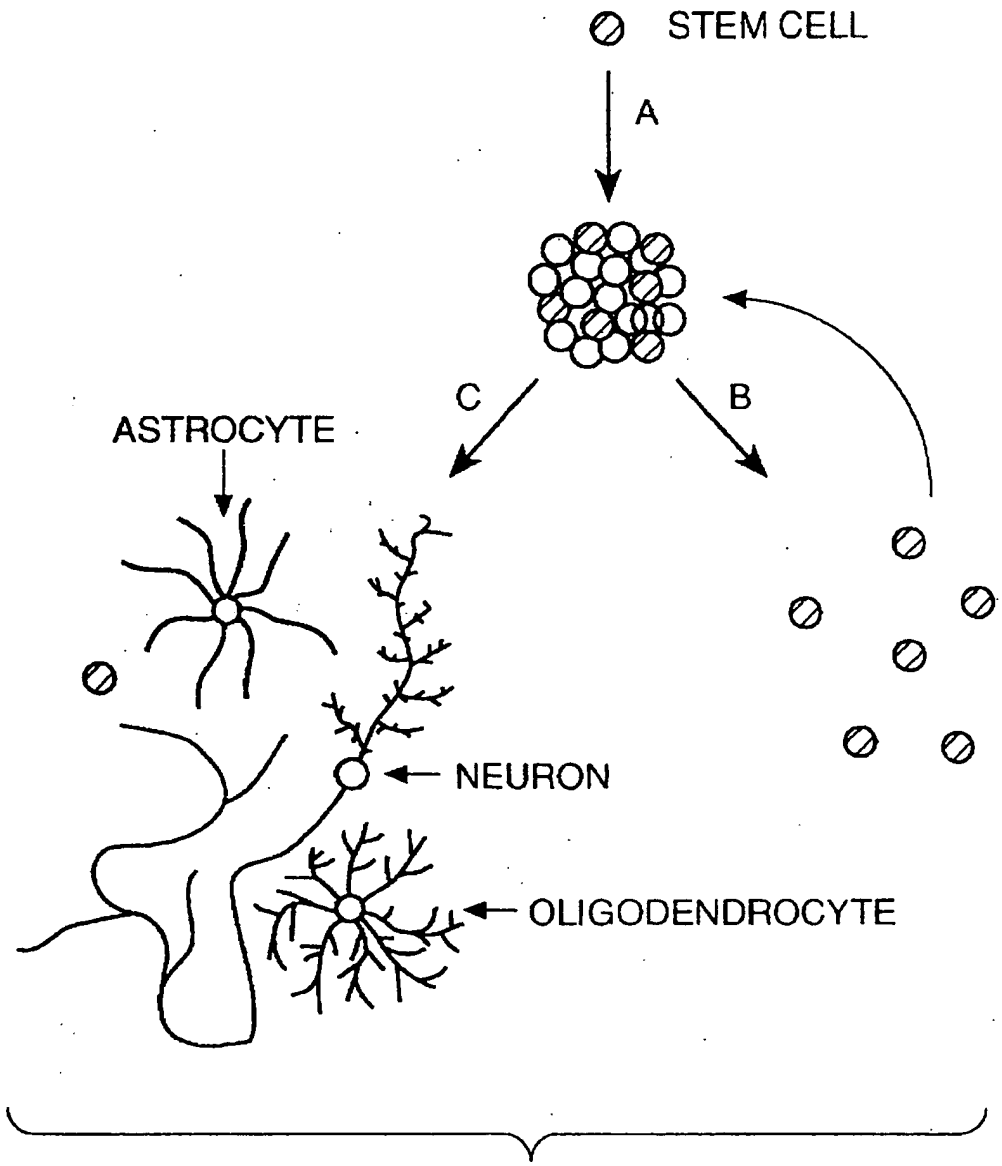

FIG._1

FIG._2A

FIG._2B

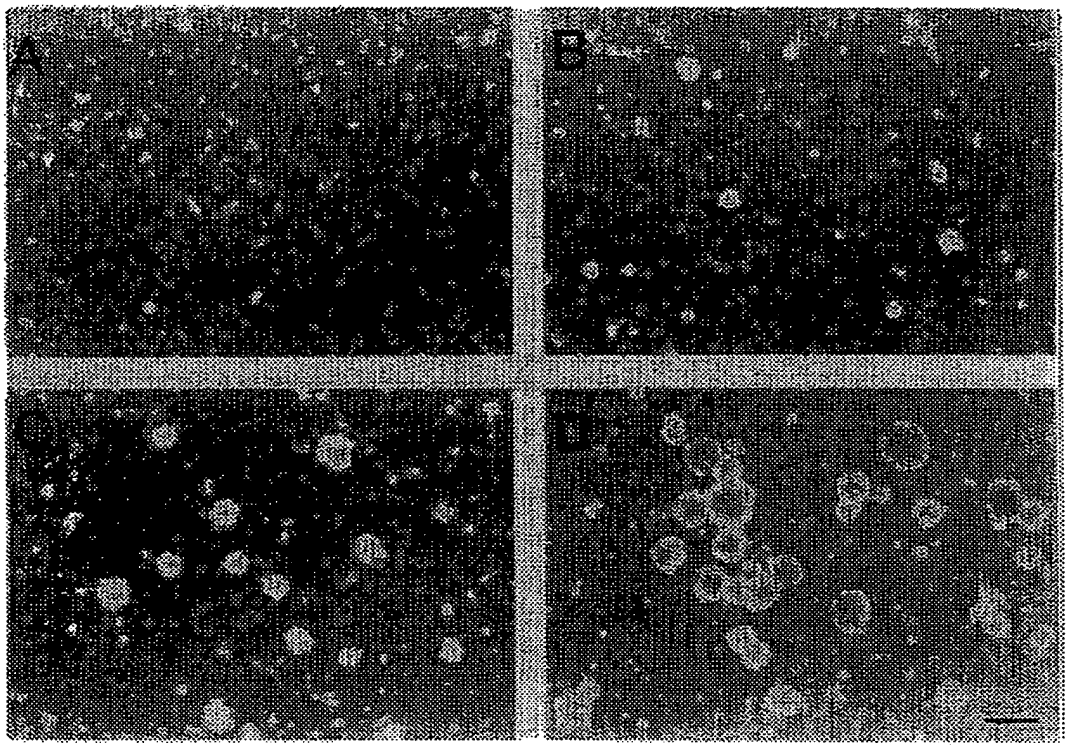

FIG._2C

FIG._2D

FIG._3A

FIG._3B

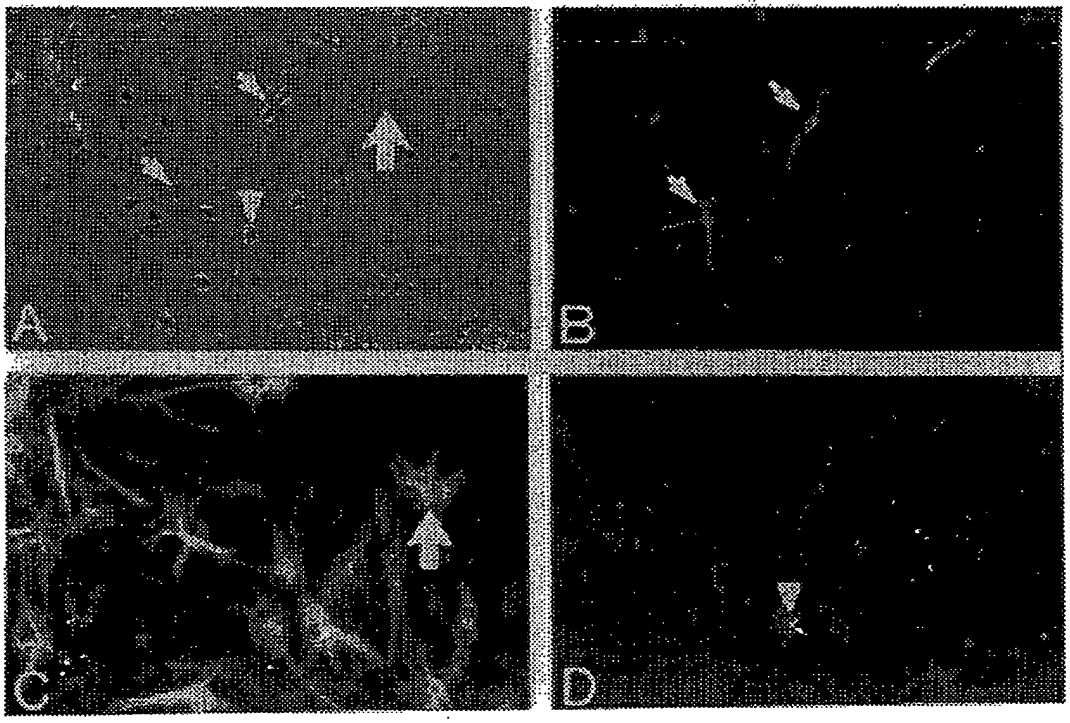

FIG._3C

FIG._3D

IN VITRO GROWTH AND PROLIFERATION OF GENETICALLY MODIFIED MULTIPOTENT NEURAL STEM CELLS AND THEIR PROGENY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Ser. No. 08/270,412, filed Jul. 5, 1994, now abandoned, which is a continuation of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned; a continuation-in-part of U.S. Ser. No. 08/385,404, filed Feb. 7. 1995, now abandoned, which is a continuation of U.S. Ser. No. 07/961,813, filed Oct. 16, 1992, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned; a continuation-in-part of U.S. Ser. No. 08/359,945, filed Dec. 20, 1994, now abandoned, which is a continuation of U.S. Ser. No. 08/221,655, filed Apr. 1, 1994, now abandoned, which is a continuation of U.S. Ser. No. 07/967, 622, filed Oct. 28, 1992, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned; a continuation-in-part of U.S. Ser. No. 08/376,062, filed Jan. 20, 1995, now abandoned, which is a continuation of U.S. Ser. No. 08/010,829, filed Jan. 29, 1993, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned; a continuation-in-part of U.S. Ser. No. 08/149,508, filed Nov. 9, 1993, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned; a continuation-in-part of U.S. Ser. No. 08/311, 099, filed Sep. 23, 1994, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned; and a continuation-in-part of U.S. Ser. No. 08/338,730, filed Nov. 14, 1994, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned.

FIELD OF THE INVENTION

This invention relates to a method for the in vitro culture and proliferation of multipotent neural stem cells, and to the use of these cells and their progeny as tissue grafts. In one aspect, this invention relates to a method for the isolation and in vitro perpetuation of large numbers of non-tumorigenic neural stem cell progeny which can be induced to differentiate and which can be used for neurotransplantation in the undifferentiated or differentiated state, into an animal to alleviate the symptoms of neurologic disease, neurodegeneration and central nervous system (CNS) trauma. In another aspect, this invention relates to a method of generating neural cells for the purposes of drug screening of putative therapeutic agents targeted at the nervous system. In another aspect, this invention also relates to a method of generating cells for autologous transplantation. In another aspect, the invention relates to a method for the in vivo proliferation and differentiation of the neural stem cell progeny in the host.

BACKGROUND OF THE INVENTION

The development of the mammalian central nervous system (CNS) begins in the early stage of fetal development and continues until the post-natal period. The mature mammalian CNS is composed of neuronal cells (neurons), and glial cells (astrocytes and oligodendrocytes).

The first step in neural development is cell birth, which is the precise temporal and spatial sequence in which stem cells and stem cell progeny (i.e daughter stem cells and progenitor cells) proliferate. Proliferating cells will give rise to neuroblasts, glioblasts and new stem cells.

The second step is a period of cell type differentiation and migration when undifferentiated progenitor cells differentiate into neuroblasts and gliolblasts which give rise to neurons and glial cells which migrate to their final positions. Cells which are derived from the neural tube give rise to neurons and glia of the CNS, while cells derived from the neural crest give rise to the cells of the peripheral nervous system (PNS). Certain factors present during development, such as nerve growth factor (NGF), promote the growth of neural cells. NGF is secreted by cells of the neural crest and stimulates the sprouting and growth of the neuronal axons.

The third step in development occurs when cells acquire specific phenotypic qualities, such as the expression of particular neurotransmitters. At this time, neurons also extend processes which synapse on their targets. Neurons are generated primarily during the fetal period, while oligodendrocytes and astrocytes are generated during the early post-natal period. By the late post-natal period, the CNS has its full complement of nerve cells.

The final step of CNS development is selective cell death, wherein the degeneration and death of specific cells, fibers and synaptic connections "fine-tune" the complex circuitry of the nervous system. This "fine-tuning" continues throughout the life of the host. Later in life, selective degeneration due to aging, infection and other unknown etiologies can lead to neurodegenerative diseases.

Unlike many other cells found in different tissues, the differentiated cells of the adult mammalian CNS have little or no ability to enter the mitotic cycle and generate new nerve cells. While it is believed that there is a limited and slow turnover of astrocytes (Korr et al., J. Comp. Neurol., 150:169, 1971) and that progenitors for oligodendrocytes (Wolsqijk and Noble, Development, 105:386, 1989) are present, the generation of new neurons does not normally occur.

Neurogenesis, the generation of new neurons, is complete early in the postnatal period. However, the synaptic connections involved in neural circuits are continuously altered throughout the life of the individual, due to synaptic plasticity and cell death. A few mammalian species (e.g. rats) exhibit the limited ability to generate new neurons in restricted adult brain regions such as the dentate gyrus and olfactory bulb (Kaplan, J. Comp. Neurol., 195:323, 1981; Bayer, N.Y. Acad. Sci., 457:163, 1985). However, this does not apply to all mammals; and the generation of new CNS cells in adult primates does not occur (Rakic, Science, 227:1054, 1985). This inability to produce new nerve cells in most mammals (and especially primates) may be advantageous for long-term memory retention; however, it is a distinct disadvantage when the need to replace lost neuronal cells arises due to injury or disease.

The low turnover of cells in the mammalian CNS together with the inability of the adult mammalian CNS to generate new neuronal cells in response to the loss of cells following injury or disease has led to the assumption that the adult mammalian CNS does not contain multipotent neural stem cells.

The critical identifying feature of a stem cell is its ability to exhibit self-renewal or to generate more of itself. The simplest definition of a stem cell would be a cell with the capacity for self-maintenance. A more stringent (but still simplistic) definition of a stem cell is provided by Potten and Loeffler (Development, 110:1001, 1990) who have defined stem cells as "undifferentiated cells capable of a)

proliferation, b) self-maintenance, c) the production of a large number of differentiated functional progeny, d) regenerating the tissue after injury, and e) a flexibility in the use of these options."

The role of stem cells is to replace cells that are lost by natural cell death, injury or disease. The presence of stem cells in a particular type of tissue usually correlates with tissues that have a high turnover of cells. However, this correlation may not always hold as stem cells are thought to be present in tissues (e.g., liver [Travis, Science, 259:1829, 1993]) that do not have a high turnover of cells.

CNS disorders encompass numerous afflictions such as neurodegenerative diseases (e.g. Alzheimer's and Parkinson's), acute brain injury (e.g. stroke, head injury, cerebral palsy) and a large number of CNS dysfunctions (e.g. depression, epilepsy, and schizophrenia). In recent years neurodegenerative disease has become an important concern due to the expanding elderly population which is at greatest risk for these disorders. These diseases, which include Alzheimer's Disease, Multiple Sclerosis (MS), Huntington's Disease, Amyotrophic Lateral Sclerosis, and Parkinson's Disease, have been linked to the degeneration of neural cells in particular locations of the CNS, leading to the inability of these cells or the brain region to carry out their intended function.

In addition to neurodegenerative diseases, acute brain injuries often result in the loss of neural cells, the inappropriate functioning of the affected brain region, and subsequent behavior abnormalities. Probably the largest area of CNS dysfunction (with respect to the number of affected people) is not characterized by a loss of neural cells but rather by an abnormal functioning of existing neural cells. This may be due to inappropriate firing of neurons, or the abnormal synthesis, release, and processing of neurotransmitters. These dysfunctions may be the result of well studied and characterized disorders such as depression and epilepsy, or less understood disorders such as neurosis and psychosis.

Degeneration in a brain region known as the basal ganglia can lead to diseases with various cognitive and motor symptoms, depending on the exact location. The basal ganglia consists of many separate regions, including the striatum (which consists of the caudate and putamen), the globus pallidus, the substantia nigra, substantia innominate, ventral pallidum, nucleus basalis of Meynert, ventral tegmental area and the subthalamic nucleus.

In the case of Alzheimer's Disease, there is a profound cellular degeneration of the forebrain and cerebral cortex. In addition, upon closer inspection, a localized degeneration in an area of the basal ganglia, the nucleus basalis of Meynert, appears to be selectively degenerated. This nucleus normally sends cholinergic projections to the cerebral cortex which are thought to participate in cognitive functions including memory.

Many motor deficits are a result of degeneration in the basal ganglia. Huntington's Chorea is associated with the degeneration of neurons in the striatum, which leads to involuntary jerking movements in the host. Degeneration of a small region called the subthalamic nucleus is associated with violent flinging movements of the extremities in a condition called ballismus, while degeneration in the putamen and globus pallidus is associated with a condition of slow writhing movements or athetosis. In the case of Parkinson's Disease, degeneration is seen in another area of the basal ganglia, the substantia nigra par compacta. This area normally sends dopaminergic connections to the dorsal striatum which are important in regulating movement. Therapy for Parkinson's Disease has centered upon restoring dopaminergic activity to this circuit.

Other forms of neurological impairment can occur as a result of neural degeneration, such as amyotrophic lateral sclerosis and cerebral palsy, or as a result of CNS trauma, such as stroke and epilepsy.

Demyelination of central and peripheral neurons occurs in a number of pathologies and leads to improper signal conduction within the nervous systems. Myelin is a cellular sheath, formed by glial cells, that surrounds axons and axonal processes that enhances various electrochemical properties and provides trophic support to the neuron. Myelin is formed by Schwann cells in the PNS and by oligodendrocytes in the CNS. Among the various demyelinating diseases MS is the most notable.

To date, treatment for CNS disorders has been primarily via the administration of pharmaceutical compounds. Unfortunately, this type of treatment has been fraught with many complications including the limited ability to transport drugs across the blood-brain barrier and the drug-tolerance which is acquired by patients to whom these drugs are administered long-term. For instance, partial restoration of dopaminergic activity in Parkinson's patients has been achieved with levodopa, which is a dopamine precursor able to cross the blood-brain barrier. However, patients become tolerant to the effects of levodopa, and therefore, steadily increasing dosages are needed to maintain its effects. In addition, there are a number of side effects associated with levodopa such as increased and uncontrollable movement.

Recently, the concept of neurological tissue grafting has been applied to the treatment of neurological diseases such as Parkinson's Disease. Neural grafts may avert the need not only for constant drug administration, but also for complicated drug delivery systems which arise due to the blood-brain barrier. However, there are limitations to this technique as well. First, cells used for transplantation which carry cell surface molecules of a differentiated cell from another host can induce an immune reaction in the host. In addition, the cells must be at a stage of development where they are able to form normal neural connections with neighboring cells. For these reasons, initial studies on neurotransplantation centered on the use of fetal cells. Perlow, et al. describe the transplantation of fetal dopaminergic neurons into adult rats with chemically induced nigrostriatal lesions in "Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system," Science 204:643–647 (1979). These grafts showed good survival, axonal outgrowth and significantly reduced the motor abnormalities in the host animals.

In both human demyelinating diseases and rodent models there is substantial evidence that demyelinated neurons are capable of remyelination in vivo. In MS, for example, it appears that there are often cycles of de- and remyelination. Similar observations in rodent demyelinating paradigms lead to the prediction that exogenously applied cells would be capable of remyelinating demyelinated axons. This approach has proven successful in a number of experimental conditions [Freidman et al., *Brain Research*, 378:142–146 (1986); Raine, et al., *Laboratory Investigation* 59:467–476 (1988); Duncan et al., *J. of Neurocytology*, 17:351–360 (1988)]. The sources of cells for some of these experiments included dissociated glial cell suspensions prepared from spinal cords (Duncan et al., supra), Schwann cell cultures prepared from sciatic nerve [Bunge et al., 1992, WO 92/03536; Blakemore and Crang, *J. Neurol. Sci.*, 70:207–223 (1985)]; cultures from dissociated brain tissue

[Blakemore and Crang, *Dev. Neurosci.* 10:1–11 (1988)], oligodendrocyte precursor cells [Gumpel et al., *Dev. Neurosci.* 11:132–139 (1989)], O-2A cells [Wolswijk et al., *Development* 109:691–608 (1990); Raff et al., *Nature* 3030:390–396 (1983); Hardy et al., *Development* 111:1061–1080 (1991)], and immortalized O-2A cell lines, [Almazan and McKay *Brain Res.* 579:234–245 (1992)].

O-2A cells are glial progenitor cells which give rise in vitro only to oligodendrocytes and type II astrocytes. Cells which appear by immunostaining in vivo to have the O-2A phenotype have been shown to successfully remyelinate demyelinated neurons in vivo, [Godfraind et al., *J. Cell Biol.* 109:2405–2416 (1989)]. Injection of a large number of O-2A cells is required to adequately remyelinate all targeted neurons in vivo, since it appears that O-2A cells (like other glial cell preparations) do not continue to divide in vivo. Although O-2A progenitor cells can be grown in culture, currently the only available isolation technique employs optic nerve as starting material. This is a low yield source, which requires a number of purification steps. There is an additional drawback that O-2A cells isolated by the available procedures are capable of only a limited number of divisions [Raff *Science* 243:1450–1455 (1989)].

Although adult CNS neurons are not good candidates for neurotransplantation, neurons from the adult PNS have been shown to survive transplantation, and to exert neurotrophic and gliotrophic effects on developing host neural tissue. One source of non-CNS neural tissue for transplantation is the adrenal medulla.

Adrenal chromaffin cells originate from the neural crest like PNS neurons, and receive synapses and produce carrier and enzyme proteins similar to PNS neurons.

Although these cells function in an endocrine manner in the intact adrenal medulla, in culture these cells lose their glandular phenotype and develop certain neural features in culture in the presence of certain growth factors and hormones [Notter, et al., "Neuronal properties of monkey adrenal medulla in vitro, *Cell Tissue Research* 244:69–76 (1986)]. When grafted into mammalian CNS, these cells survive and synthesize significant quantities of dopamine which can interact with dopamine receptors in neighboring areas of the CNS.

In U.S. Pat. No. 4,980,174, transplantation of monoamine-containing cells isolated from adult rat pineal gland and adrenal medulla into rat frontal cortex led to the alleviation of learned helplessness, a form of depression in the host. In U.S. Pat. No. 4,753,635, chromaffin cells and adrenal medullary tissue derived from steers were implanted into the brain stem or spinal cord of rats and produced analgesia when the implanted tissue or cell was induced to release nociceptor interacting substances (i.e. catecholamines such as dopamine). Adrenal medullary cells have been autologously grafted into humans, and have survived, leading to mild to moderate improvement in symptoms (Watts, et al., "Adrenal-caudate transplantation in patients with Parkinson's Disease (PD):1-year follow-up," *Neurology* 39 Suppl 1: 127 [1989], Hurtig, et al., "Postmortem analysis of adrenal-medulla-to-caudate autograft in a patient with Parkinson's Disease," *Annals of Neurology* 25: 607–614 [1989]). However, adrenal cells do not obtain a normal neural phenotype, and are therefore probably of limited use for transplants where synaptic connections must be formed.

Another source of tissue for neurotransplantation is from cell lines. Cell lines are immortalized cells which are derived either by transformation of normal cells with an oncogene (Cepko, "Immortalization of neural cells via retrovirus-mediated oncogene transduction," *Ann. Rev. Neurosci.* 12:47–65 [1989]) or by the culturing of cells with altered growth characteristics in vitro (Ronnett, et al., "Human cortical neuronal cell line: Establishment from a patient with unilateral megalencephaly," *Science* 248:603–605 [1990]). Such cells can be grown in culture in large quantities to be used for multiple transplantations. Some cell lines have been shown to differentiate upon chemical treatment to express a variety of neuronal properties such as neurite formation, excitable membranes and synthesis of neurotransmitters and their receptors. Furthermore, upon differentiation, these cells appear to be amitotic, and therefore noncancerous. However, the potential for these cells to induce adverse immune responses, the use of retroviruses to immortalize cells, the potential for the reversion of these cells to an amitotic state, and the lack of response of these cells to normal growth-inhibiting signals make cell lines less than optimal for widespread use.

Another approach to neurotransplantation involves the use of genetically engineered cell types or gene therapy. Using this method, a foreign gene or transgene can be introduced into a cell which is deficient in a particular enzymatic activity, thereby allowing the cell to express the gene. Cells which now contain the transferred gene can be transplanted to the site of neurodegeneration, and provide products such as neurotransmitters and growth factors (Rosenberg, et al., "Grafting genetically modified cells to the damaged brain: Restorative effects of NGF Expression," *Science* 242:1575–1578, [1988]) which may function to alleviate some of the symptoms of degeneration. However, there still exists a risk of inducing an immune reaction using currently available cell lines. In addition, these cells may also not achieve normal neuronal connections with the host tissue.

Genetically modified cells have been used in neurological tissue grafting in order to replace lost cells which normally produce a neurotransmitter. For example, fibroblasts have been genetically modified with a retroviral vector containing a cDNA for tyrosine hydroxylase, which allows them to produce dopamine, and implanted into animal models of Parkinson's Disease (Gage et al., U.S. Pat. No. 5,082,670).

While the use of genetically modified fibroblasts to treat CNS disorders has shown promise in improving some behavioral deficits in animal models of Parkinson's Disease, and represents a novel approach to supplying a needed transmitter to the CNS, it suffers from several significant drawbacks as a treatment for Parkinson's Disease and in general as a therapeutic approach for treating neurodegenerative diseases and brain injury. First, the CNS is primarily composed of three cell types—neurons, astrocytes and oligodendrocytes. The implantation of a foreign cell such as a fibroblast into the CNS and its direct and indirect effects on the functioning of the host cells has yet to be studied. However, it is likely that the expression of membrane bound factors and the release of soluble molecules such as growth factors and proteases will alter the normal behavior of the surrounding tissue. This may result in the disruption of neuronal firing patterns either by a direct action on neurons or by an alteration in the normal functioning of glial cells.

Another concern that arises when fibroblasts are implanted into the CNS is the possibility that the implanted cells may lead to tumor formation because the intrinsic inhibition of fibroblast division is poorly controlled. Instead, extrinsic signals play a major role in controlling the number of divisions the cell will undergo. The effect of the CNS environment on the division of implanted fibroblasts and the

high probability of a fibroblastic tumor formation has not been studied in the long-term.

A third concern in transplanting fibroblasts into the CNS is that fibroblasts are unable to integrate with the CNS cells as astrocytes, oligodendrocytes, or neurons do. Fibroblasts are intrinsically limited in their ability to extend neuronal-like processes and form synapses with host tissue. Hence, although the genetic modification and implantation of fibroblasts into the CNS represents an improvement over the current technology for the delivery of certain molecules to the CNS, the inability of fibroblasts to integrate and function as CNS tissue, their potential negative effects on CNS cells, and their limited intrinsic control of proliferation limits their practical usage for implantation for the treatment of acute or chronic CNS injury or disease.

A preferred tissue for genetic modification and implantation would be CNS cells—neurons, astrocytes, or oligodendrocytes. One source of CNS cells is from human fetal tissue. Several studies have shown improvements in patients with Parkinson's Disease after receiving implants of fetal CNS tissue. Implants of embryonic mesencephalic tissue containing dopamine cells into the caudate and putamen of human patients was shown by Freed et al. (*N Engl J Med* 327:1549–1555 (1992)) to offer long-term clinical benefit to some patients with advanced Parkinson's Disease. Similar success was shown by Spencer et al. (*N Engl J Med* 327:1541–1548 (1992)). Widner et al. (*N Engl J Med* 327:1556–1563 (1992)) have shown long-term functional improvements in patients with MPTP-induced Parkinsonism that received bilateral implantation of fetal mesencephalic tissue.

While the studies noted above are encouraging, the use of large quantities of aborted fetal tissue for the treatment of disease raises ethical considerations and political obstacles. There are other considerations as well. Fetal CNS tissue is composed of more than one cell type, and thus is not a well-defined source of tissue. In addition, there are serious doubts as to whether an adequate and constant supply of fetal tissue would be available for transplantation. For example, in the treatment of MPTP-induced Parkinsonism (Widner supra) tissue from 6 to 8 fetuses were used for implantation into the brain of a single patient. There is also the added problem of the potential for contamination during fetal tissue preparation. Moreover, the tissue may already be infected with a bacteria or virus, thus requiring expensive diagnostic testing for each fetus used. However, even diagnostic testing might not uncover all infected tissue. For example, the diagnosis of HIV-free tissue is not guaranteed because antibodies to the virus are generally not present until several weeks after infection.

While currently available transplantation approaches represent a significant improvement over other available treatments for neurological disorders, they suffer from significant drawbacks. The inability in the prior art of the transplant to fully integrate into the host tissue, and the lack of availability of cells in unlimited amounts from a reliable source for grafting are, perhaps, the greatest limitations of neurotransplantation.

It would be more preferable to have a well-defined, reproducible source of neural tissue for transplantation that is available in unlimited amounts. Since adult neural tissue undergoes minimal division, it does not readily meet these criteria. While astrocytes retain the ability to divide and are probably amenable to infection with foreign genes, their ability to form synapses with neuronal cells is limited and consequently so is their extrinsic regulation of the expression and release of the foreign gene product.

Oligodendrocytes suffer from some of the same problems. In addition, mature oligodendrocytes do not divide, limiting the infection of oligodendrocytes to their progenitor cells (e.g. O2A cells). However, due to the limited proliferative ability of oligodendrocyte progenitors, the infection and harvesting of these cells does not represent a practical source.

The infection of neurons with foreign genes and implantation into the CNS would be ideal due to their ability to extend processes, make synapses and be regulated by the environment. However, differentiated neurons do not divide and transfection with foreign genes by chemical and physical means is not efficient, nor are they stable for long periods of time. The infection of primary neuronal precursors with retroviral vectors in vitro is not practical either because neuroblasts are intrinsically controlled to undergo a limited number of divisions making the selection of a large number of neurons, that incorporate and express the foreign gene, nearly impossible. The possibility of immortalizing the neuronal precursors by retroviral transfer of oncogenes and their subsequent infection of a desired gene is not preferred due to the potential for tumor formation by the implanted cells.

In addition to the need for a well-defined, reproducible source of neural cells available in unlimited amounts for transplantation purposes, a similar need exists for drug screening purposes and for the study of CNS function, dysfunction, and development. The mature human nervous system is composed of billions of cells that are generated during development from a small number of precursors located in the neural tube. Due to the complexity of the mammalian CNS, the study of CNS developmental pathways, as well as alterations that occur in adult mammalian CNS due to dysfunction, has been difficult. Such areas would be better studied using relatively simple models of the CNS under defined conditions.

Generally, two approaches have been taken for studying cultured CNS cells: the use of primary neural cultures; and the use of neural cell lines. Primary mammalian neural cultures can be generated from nearly all brain regions providing that the starting material is obtained from fetal or early post-natal animals. In general, three types of cultures can be produced, enriched either in neurons, astrocytes, or oligodendrocytes. Primary CNS cultures have proven valuable for discovering many mechanisms of neural function and are used for studying the effects of exogenous agents on developing and mature cells. While primary CNS cultures have many advantages, they suffer from two primary drawbacks. First, due to the limited proliferative ability of primary neural cells, new cultures must be generated from several different animals. While great care is usually taken to obtain tissue at identical states of development and from identical brain regions, it is virtually impossible to generate primary cultures that are identical. Hence, there exists a significant degree of variability from culture to culture.

A second disadvantage of primary cultures is that the tissue must be obtained from fetuses or early post-natal animals. If primary cultures are to be performed on a regular basis, this requires the availability of a large source of starting material. While this is generally not a problem for generating primary cultures from some species (e.g. rodents), it is for others (e.g. primates). Due to the limited supply and ethical concerns, the culturing of primary cells from primates (both human and non-human) is not practical.

Due to the limited proliferative ability of primary neural cells, the generation of a large number of homogenous cells

for studies of neural function, dysfunction, and drug design/screening has previously not been achieved. Therefore, homogenous populations of cells that can generate a large number of progeny for the in vitro investigation of CNS function has been studied by the use of cell lines. The generation of neural cell lines can be divided into two categories: 1) spontaneously occurring tumors, and 2) custom-designed cell lines.

Of the spontaneously occurring tumors, probably the most studied cell line for neurobiology is the rat pheochromocytoma (PC12) cells that can differentiate into sympathetic-like neurons in response to NGP. These cells have proven to be a useful model for studying mechanisms of neural development and alterations (molecular and cellular) in response to growth factors. Neuroblastoma and glioma cell lines have been used to study neuronal and glial functioning [Liles, et al., *J. Neurosci.* 7, 2556–2563 (1987); Nister et al. *Cancer Res.* 48(14) 3910 (1988)]. Embryonal carcinoma cells are derived from teratoma tumors of fetal germ cells and have the ability to differentiate into a large number of non-neural cell types with some lines (e.g. P19 cells) [Jones-Villeneuve et al. *J. Cell Biol.* 94, 253–262 (1982)] having the ability to differentiate into neural cells [(McBurney et al. *J. Neurosci.* 8(3) 1063–73 (1993)]. A human teratocarcinoma-derived cell line, NTera 2/cl.D1, with a phenotype resembling CNS neuronal precursor cells, can be induced to differentiate in the presence of retinoic acid. However, the differentiated cells are restricted to a neuronal phenotype [Pleasure and Lee *J. Neurosci. Res.* 35: 585–602 (1993)]. While these types of cell lines are able to generate a large number of cells for screening the effects of exogenous agents on cell survival or function, the limited number of these types of lines, the limited number of phenotypes that they are able to generate and the unknown nature of their immortalization (which may effect the function of the cells in an undefined manner) makes these types of cell lines less than ideal for in vitro models of neural function and discovery of novel therapeutics.

An alternative approach to spontaneously occurring cell lines is the intentional immortalization of a primary cell by introducing an oncogene that alters the genetic make-up of the cell thereby inducing the cell to proliferate indefinitely. This approach has been used by many groups to generate a number of interesting neural cell lines [(Bartlett et al. *Proc. Nat. Acad. Sci.* 85(9) 3255–3259 (1988); Frederiksen et al. *Neuron* 1, 439–448 (1988); Trotter et al. *Oncogene* 4: 457–464 (1989); Ryder et al. *J. Neurobiol.* 21: 356–375 (1980); Murphy et al. *J. Neurobiol* 22: 522–535 (1991); Almazan and McKay et al. *Brain Res.* 579: 234–245 (1992)]. While these lines may prove useful for studying the decisions that occur during cell determination and differentiation, and for testing the effects of exogenous agents, they suffer from several drawbacks. First, the addition of an oncogene that alters the proliferative status of a cell may affect other properties of the cell (oncogenes may play other roles in cells besides regulating the cell cycle). This is well illustrated in a study by Almazan and McKay, supra, and their immortalization of an oligodendrocyte precursor from the optic nerve which is unable to differentiate into type II astrocytes (something that normal optic nerve oligodendrocyte precursors can do). The authors suggest the presence of the immortalizing antigen may alter the cells ability to differentiate into astrocytes.

Another drawback to using intentionally immortalized cells results from the fact that the nervous system is composed of billions of cells and possibly thousands of different cell types, each with unique patterns of gene expression and responsiveness to their environment. A custom-designed cell line is the result of the immortalization of a single progenitor cell and its clonal expansion. While a large supply of one neural cell type can be generated, this approach does not take into account cellular interactions between different cell types. In addition, while it is possible to immortalize cells from a given brain region, immortalization of a desired cell is not possible due to the lack of control over which cells will be altered by the oncogene. Hence, while custom designed cell lines offer a few advantages over spontaneously occurring tumors, they suffer from several drawbacks and are less than ideal for understanding CNS function and dysfunction.

Therefore, in view of the aforementioned deficiencies attendant with prior art methods of neural cell culturing, transplantation, and CNS models, a need exists in the art for a reliable source of unlimited numbers of undifferentiated neural cells for neurotransplantation and drug screening which are capable of differentiating into neurons, astrocytes, and oligodendrocytes. Preferably cellular division in such cells from such a source would be epigenetically regulated and a suitable number of cells could be efficiently prepared in sufficient numbers for transplantation. The cells should be suitable in autografts, xenografts, and allografts without a concern for tumor formation. There exists a need for the isolation, perpetuation and transplantation of autologous neural cells from the juvenile or adult brain that are capable of differentiating into neurons and glia.

A need also exists for neural cells, capable of differentiating into neurons, astrocytes and oligodendrocytes that are capable of proliferation in vitro and thus amenable to genetic modification techniques.

Additionally, there exists a need for the repair of damaged neural tissue in a relatively non-invasive fashion, that is by inducing neural cells to proliferate and differentiate into neurons, astrocytes, and oligodendrocytes in vivo, thereby averting the need for transplantation.

Accordingly, a major object of the present invention is to provide a reliable source of an unlimited number of neural cells for neurotransplantation that are capable of differentiating into neurons, astrocytes, and oligodendrocytes.

It is another object of the present invention to provide a method for the in vitro proliferation of neural stem cells from embryonic, juvenile and adult brain tissue, to produce unlimited numbers of precursor cells available for transplantation that are capable of differentiating into neurons, astrocytes, and oligodendrocytes. A further object of the invention is to provide methods for inducing neural cells to proliferate and differentiate in vivo, thereby averting the need for neurotransplantation.

A still further object of the invention is to provide a method of generating large numbers of normal neural cells for the purpose of screening putative therapeutic agents targeted at the nervous system and for models of CNS development, function, and dysfunction.

SUMMARY OF THE INVENTION

This invention provides in one aspect a composition for inducing the proliferation of a multipotent neural stem cell comprising a culture medium supplemented with at least one growth factor, preferably epidermal growth factor or transforming growth factor alpha.

The invention also provides a method for the in vitro proliferation and differentiation of neural stem cells and stem cell progeny comprising the steps of (a) isolating the cell from a mammal, (b) exposing the cell to a culture medium containing a growth factor, (c) inducing the cell to

proliferate, and (d) inducing the cell to differentiate. Proliferation and perpetuation of the neural stem cell progeny can be carried out either in suspension cultures, or by allowing cells to adhere to a fixed substrate. Proliferation and differentiation can be done before or after transplantation, and in various combinations of in vitro or in vivo conditions, including (1) proliferation and differentiation in vitro, then transplantation, (2) proliferation in vitro, transplantation, then further proliferation and differentiation in vivo, and (3) proliferation in vitro, transplantation and differentiation in vivo.

The invention also provides for the proliferation and differentiation of the progenitor cells in vivo, which can be done directly in the host without the need for transplantation.

The invention also provides a method for the in vivo transplantation of neural stem cell progeny, treated as in any of (1) through (3) above, which comprises implanting, into a mammal, these cells which have been treated with at least one growth factor.

Furthermore, the invention provides a method for treating neurodegenerative diseases comprising administering to a mammal neural stem cell progeny which have been treated as in any of (1) through (3), and induced to differentiate into neurons and/or glia.

The invention also provides a method for treating neurodegenerative disease comprising stimulating in vivo mammalian CNS neural stem cells to proliferate and the neural stem cell progeny to differentiate into neurons and/or glia. The invention also provides a method for the transfection of neural stem cells and stem cell progeny with vectors which can express the gene products for growth factors, growth factor receptors, and peptide neurotransmitters, or express enzymes which are involved in the synthesis of neurotransmitters, including those for amino acids, biogenic amines and neuropeptides, and for the transplantation of these transfected cells into regions of neurodegeneration.

In a still further aspect, the invention provides a method for the screening of potential neurologically therapeutic pharmaceuticals using neural stem cell progeny which have been proliferated in vitro.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A–C: Diagram Illustrating the Proliferation of a Multipotent Neural Stem Cell

(A) In the presence of a proliferation-inducing growth factor the stem cell divides and gives rise to a sphere of undifferentiated cells composed of more stem cells and progenitor cells. (B) When the clonally derived sphere of undifferentiated cells is dissociated and plated as single cells, on a non-adhesive substrate and in the presence of a proliferation-inducing growth factor, each stem cell will generate a new sphere. (C) If the spheres are cultured in conditions that allow differentiation, the progenitor cells differentiate into neurons, astrocytes and oligodendrocytes.

FIG. 2A–D: Proliferation Of Epidermal Growth Factor (EGF) Responsive Cells

After 2 days in vitro EGF-responsive cells begin to proliferate (FIG. 2A). After 4 days in vitro small clusters of cells known as neurospheres are apparent (FIG. 2B). The neurospheres of continuously proliferating cells continue to grow in size (FIG. 2C) until they lift off the substrate and float in suspension (FIG. 2D). At this stage, the floating spheres can be easily removed, dissociated into single cells and, in the presence of EGF, proliferation can be re-initiated. (Bar: 50 μm).

FIG. 3A–D: Differentiation Of Cells From Single EGF-Generated Spheres Into Neurons, Astrocytes, And Oligodendrocytes

Triple-label immunocytochemistry with antibodies to microtubule associated protein (MAP-2), glial fibrillary acidic protein (GFAP), and O4 (a cell surface antigen) are used to detect the presence of neurons (FIG. 3B), astrocytes (FIG. 3C) and oligodendrocytes (FIG. 3D), respectively, from an EGF-generated, stem cell-derived neurosphere (FIG. 3A) derived from primary culture. (Bar: 50 μm).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for inducing multipotent neural stem cells from fetal, juvenile, or adult mammalian tissue to proliferate in vitro or in vivo (i.e. in situ), to generate large numbers of neural stem cell progeny capable of differentiating into neurons, astrocytes, and oligodendrocytes. Methods for differentiation of the neural stem cell progeny are also provided. The induction of proliferation and differentiation of neural stem cells can be done either by culturing the cells in suspension or on a substrate onto which they can adhere. Alternatively, proliferation and differentiation of neural stem cells can be induced, under appropriate conditions, in the host in the following combinations: (1) proliferation and differentiation in vitro, then transplantation, (2) proliferation in vitro, transplantation, then further proliferation and differentiation in vivo, (3) proliferation in vitro, transplantation and differentiation in vivo, and (4) proliferation and differentiation in vivo. Proliferation and differentiation in vivo (i.e. in situ) can involve a non-surgical approach that coaxes neural stem cells to proliferate in vivo with pharmaceutical manipulation. Thus, the invention provides a means for generating large numbers of undifferentiated and differentiated neural cells for neurotransplantation into a host in order to treat neurodegenerative disease and neurological trauma, for non-surgical methods of treating neurodegenerative disease and neurological trauma, and for drug-screening applications.

Multipotent Neural Stem Cells

Neurobiologists have used various terms interchangeably to describe the undifferentiated cells of the CNS. Terms such as "stem cell", "precursor cell" and "progenitor cell" are commonly used in the scientific literature. However, there are different types of undifferentiated neural cells, with differing characteristics and fates. U.S. Ser. No. 08/270,412 which is a continuation application of U.S. Ser. No. 07/726,812, termed the cells obtained and proliferated using the methods of Examples 1–4 below "progenitor cells". The terminology used for undifferentiated neural cells has evolved such that these cells are now termed "neural stem cells". U.S. Ser. No. 08/270,412 defines the "progenitor" cell proliferated in vitro to mean "an oligopotent or multipotent stem cell which is able to divide without limit and under specific conditions can produce daughter cells which terminally differentiate into neurons and glia." The capability of a cell to divide without limit and produce daughter cells which terminally differentiate into neurons and glia are stem cell characteristics. Accordingly, as used herein, the cells proliferated using the methods described in Examples 1–4 are termed "neural stem cells". A neural stem cell is an undifferentiated neural cell that can be induced to proliferate using the methods of the present invention. The neural stem cell is capable of self-maintenance, meaning that with each cell division, one daughter cell will also be a stem cell. The

non-stem cell progeny of a neural stem cell are termed progenitor cells. The progenitor cells generated from a single multipotent neural stem cell are capable of differentiating into neurons, astrocytes (type I and type II) and oligodendrocytes. Hence, the neural stem cell is "multipotent" because its progeny have multiple differentiative pathways.

The term "neural progenitor cell", as used herein, refers to an undifferentiated cell derived from a neural stem cell, and is not itself a stem cell. Some progenitor cells can produce progeny that are capable of differentiating into more than one cell type. For example, an O-2A cell is a glial progenitor cell that gives rise to oligodendrocytes and type II astrocytes, and thus could be termed a "bipotential" progenitor cell. A distinguishing feature of a progenitor cell is that, unlike a stem cell, it has limited proliferative ability and thus does not exhibit self-maintenance. It is committed to a particular path of differentiation and will, under appropriate conditions, eventually differentiate into glia or neurons.

The term "precursor cells", as used herein, refers to the progeny of neural stem cells, and thus includes both progenitor cells and daughter neural stem cells.

Neural stem cell progeny can be used for transplantation into a heterologous, autologous, or xenogeneic host. Multipotent neural stem cells can be obtained from embryonic, post-natal, juvenile or adult neural tissue. The neural tissue can be obtained from any animal that has neural tissue such as insects, fish, reptiles, birds, amphibians, mammals and the like. The preferred source neural tissue is from mammals, preferably rodents and primates, and most preferably, mice and humans.

In the case of a heterologous donor animal, the animal may be euthanized, and the neural tissue and specific area of interest removed using a sterile procedure. Areas of particular interest include any area from which neural stem cells can be obtained that will serve to restore function to a degenerated area of the host's nervous system, particularly the host's CNS. Suitable areas include the cerebral cortex, cerebellum, midbrain, brainstem, spinal cord and ventricular tissue, and areas of the PNS including the carotid body and the adrenal medulla. Preferred areas include regions in the basal ganglia, preferably the striatum which consists of the caudate and putamen, or various cell groups such as the globus pallidus, the subthalamic nucleus, the nucleus basalis which is found to be degenerated in Alzheimer's Disease patients, or the substantia nigra pars compacta which is found to be degenerated in Parkinson's Disease patients. Particularly preferred neural tissue is obtained from ventricular tissue that is found lining CNS ventricles and includes the subependyma. The term "ventricle" refers to any cavity or passageway within the CNS through which cerebral spinal fluid flows. Thus, the term not only encompasses the lateral, third, and fourth ventricles, but also encompasses the central canal, cerebral aqueduct, and other CNS cavities.

Human heterologous neural stem cells may be derived from fetal tissue following elective abortion, or from a post-natal, juvenile or adult organ donor. Autologous neural tissue can be obtained by biopsy, or from patients undergoing neurosurgery in which neural tissue is removed, for example, during epilepsy surgery, temporal lobectomies and hippocampalectomies. Neural stem cells have been isolated from a variety of adult CNS ventricular regions, including the frontal lobe, conus medullaris, thoracic spinal cord, brain stem, and hypothalamus, and proliferated in vitro using the methods detailed herein. In each of these cases, the neural stem cell exhibits self-maintenance and generates a large number of progeny which include neurons, astrocytes and oligodendrocytes.

Normally, the adult mammalian CNS is mitotically quiescent in vivo with the exception of the subependymal region lining the lateral ventricles in the forebrain. This region contains a subpopulation of constitutively proliferating cells with a cell cycle time of 12.7 hours. BrdU and retroviral labeling of the proliferating cells reveal that none of the newly generated cells differentiate into mature neurons or glia nor do they migrate into other CNS regions (Morshead and Van der Kooy, supra).

The continual proliferation and maintenance of a constant number of cells within the subependyma is explained by two mechanisms. The death of one of the daughter cells after each division maintains the proliferating population at a constant number. The constitutively dividing population eventually dies out (and hence is not a stem cell population) however, a subpopulation of relatively quiescent cells within the subependyma is able to repopulate the constitutively dividing population. This stem cell-like mode of maintaining the proliferative subependymal population is analogous to other tissues where cells have a short life span and are repopulated by a subpopulation of relatively quiescent cells referred to as stem cells.

As detailed in Example 27, experiments utilizing retrovirus infection of constituitively proliferating cells in vivo and subsequent β-galactosidase (β-gal) reporter gene expression as a non-diluting marker show that with increasing adult mice survival times (of up to 28 days post retrovirus infection) there is a progressive loss of β-gal positive subependymal cells. Relative to 1 day survival animals, 6 days following retrovirus injection there is a 45% loss of β-gal positive cells and 28 days following retrovirus infection there is a 97% loss. Using nested polymerase chain reaction (PCR) to identify single cells containing retroviral DNA it was determined that the loss of β-gal expressing cells is due to the loss of the retrovirally infected cells through cell death, not due to the turn-off of β-gal expression.

Intraperitoneal injections of BrdU (a thymidine analog that is incorporated into the DNA of dividing cells) reveal that 33% of the cells within some regions of the subependyma make up the normally constituitively dividing population (see Morshead and van der Kooy, *J. Neurosci.* 12:249 (1992)). The number of BrdU labelled cells decreases over time. By 30 days after BrdU labeling, only 3% of the dividing cells are still labelled. The heavy labeling of only a small number of cells 30 days after BrdU injections demonstrates that although the labelled cells were dividing at the time of the injections they were relatively quiescent for the 30 day period. This suggests that these few labeled cells are stem cells rather than cells of the constitutively proliferating population.

The above two examples support the hypothesis that the maintenance of the constant number of proliferating subependymal cells seen throughout adult life requires the presence of a relatively quiescent stem cell that proliferates sporadically to replenish the constitutively proliferating population and to self-renew.

As detailed in Example 24, the constitutively dividing subependymal cells can be killed off by injecting high doses of radioactive thymidine for the duration of the cell cycle at intervals less than S-phase duration. At one day post-kill the proliferating population is 10% of controls and by 8 days the proliferating population is back to control levels. If the

replenished population is due to the recruitment of normally quiescent stem cells into the proliferative mode, then a second kill at the time that stem cells are generating progeny to repopulate the subependyma should alter the number of cells within the constitutively proliferating population. When a second kill is done 2 days after the initial kill, 8 days later the constitutively proliferating population is only 45% of the control values (animals receiving no thymidine kill treatment) or animals that received only one kill at day 0 (the time of the first kill). The reduction in the number of proliferative cells in the subependyma is maintained at 63% even at 31 days after the second kill. When a second kill is done on day 4, the proliferating population returns to 85% of control values 8 days later. These results suggest that the normally quiescent stem cell is recruited into the proliferative mode within the first two days after the initial kill and that by 4 days the stem cell no longer needs to be recruited to repopulate the subependyma.

As detailed in Example 26 below, an experiment was performed to determine whether the in vitro stem cell is derived from the constitutively proliferating population or from the quiescent population. Animals were treated in one of the following ways:

Group 1. Control

High dose of radioactive thymidine were given on:

Group 2. day 0

Group 3. day 0 and day 2

Group 4. day 0 and day 4

16 to 20 following the last injection animals were killed and stem cells isolated from the striatum (including the subependymal region) via the methods described in Example 2 below.

In groups 2–4 the constitutively proliferating population was killed. In group 3 stem cells that are recruited into the cell cycle to repopulate the subependymal proliferating cells were also killed.

Number of Neurospheres produced in vitro:

Group 1. 100% (Control)

Group 2. 100%

Group 3. 45%

Group 4. 85%

These results demonstrate that when you eliminate nearly all of the constitutively proliferating cells in the subependyma this does not affect the number of stem cells that can be isolated and proliferated in vitro (group 1 vs. group 2 and 4). However, when the normally quiescent cells are killed when they are recruited to repopulate the subependyma (as with group 3) the number of stem cells that can be isolated in vitro is significantly reduced (group 3 vs. group 1 and 2). By 4 days after the first kill most of the stem cells themselves are no longer turning over and as a result are not killed by the second series of tritiated thymidine injections (hence, only a 15% reduction [group 4] compared to 55% reduction [group 3]).

The above results demonstrate that, in adult, the stem cells which are proliferated in vitro are derived from the quiescent population of subependymal cells in vivo. This also explains why stem cells can be derived from CNS ventricular regions, other than the forebrain, which do not have a subpopulation of constitutively proliferating cells.

In Vitro Proliferation of Neural Stem Cells

Cells can be obtained from donor tissue by dissociation of individual cells from the connecting extracellular matrix of the tissue. Tissue from a particular neural region is removed from the brain using a sterile procedure, and the cells are dissociated using any method known in the art including treatment with enzymes such as trypsin, collagenase and the like, or by using physical methods of dissociation such as with a blunt instrument. Dissociation of fetal cells can be carried out in tissue culture medium, while a preferable medium for dissociation of juvenile and adult cells is low Ca^{2+} artificial cerebral spinal fluid (aCSF). Regular aCSF contains 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose. Low Ca^{2+} aCSF contains the same ingredients except for $MgCl_2$ at a concentration of 3.2 mM and $CaCl_2$ at a concentration of 0.1 mM. Dissociated cells are centrifuged at low speed, between 200 and 2000 rpm, usually between 400 and 800 rpm, and then resuspended in culture medium. The neural cells can be cultured in suspension or on a fixed substrate. However, substrates tend to induce differentiation of the neural stem cell progeny. Thus, suspension cultures are preferred if large numbers of undifferentiated neural stem cell progeny are desired. Cell suspensions are seeded in any receptacle capable of sustaining cells, particularly culture flasks, culture plates or roller bottles, and more particularly in small culture flasks such as 25 cm^2 culture flasks. Cells cultured in suspension are resuspended at approximately 5×10^4 to 2×10^5 cells/ml, preferably 1×10^5 cells/mL. Cells plated on a fixed substrate are plated at approximately $2–3\times10^3$ cells/cm^2, preferably 2.5×10^5 cells/cm^2.

The dissociated neural cells can be placed into any known culture medium capable of supporting cell growth, including HEM, DMEM, RPMI, F-12, and the like, containing supplements which are required for cellular metabolism such as glutamine and other amino acids, vitamins, minerals and useful proteins such as transferrin and the like. Medium may also contain antibiotics to prevent contamination with yeast, bacteria and fungi such as penicillin, streptomycin, gentamicin and the like. In some cases, the medium may contain serum derived from bovine, equine, chicken and the like. However, a preferred embodiment for proliferation of neural stem cells is to use a defined, serum-free culture medium, as serum tends to induce differentiation and contains unknown components (i.e. is undefined). A defined culture medium is also preferred if the cells are to be used for transplantation purposes. A particularly preferable culture medium is a defined culture medium comprising a mixture of DMEM, F12, and a defined hormone and salt mixture. This culture medium is referred to herein as "Complete Medium" and is described in detail in Example 3.

Conditions for culturing should be close to physiological conditions. The pH of the culture medium should be close to physiological pH, preferably between pH 6–8, more preferably between about pH 7 to 7.8, with pH 7.4 being most preferred. Physiological temperatures range between about 30° C. to 40° C. Cells are preferably cultured at temperatures between about 32° C. to about 38° C., and more preferably between about 35° C. to about 37° C.

The culture medium is supplemented with at least one proliferation-inducing growth factor. As used herein, the term "growth factor" refers to a protein, peptide or other molecule having a growth, proliferative, differentiative, or trophic effect on neural stem cells and/or neural stem cell progeny. Growth factors which may be used for inducing proliferation include any trophic factor that allows neural stem cells and precursor cells to proliferate, including any molecule which binds to a receptor on the surface of the cell to exert a trophic, or growth-inducing effect on the cell. Preferred proliferation-inducing growth factors include EGF, amphiregulin, acidic fibroblast growth factor (aFGF or FGF-1), basic fibroblast growth factor (bFGF or FGF-2), transforming growth factor alpha (TGFα), and combinations thereof.

Preferred proliferation-inducing growth factors include EGF and TGFα. A preferred combination of proliferation-inducing growth factors is EGF or TGFC with FGF-1 or FGF-2. Growth factors are usually added to the culture medium at concentrations ranging between about 1 fg/ml to 1 mg/ml. Concentrations between about 1 to 100 ng/ml are usually sufficient. Simple titration experiments can be easily performed to determine the optimal concentration of a particular growth factor.

In addition to proliferation-inducing growth factors, other growth factors may be added to the culture medium that influence proliferation and differentiation of the cells including NGF, platelet-derived growth factor (PDGF), thyrotropin releasing hormone (TRH), transforming growth factor betas (TGFβs), insulin-like growth factor (IGF_{-1}) and the like.

Within 3–4 days in the presence of a proliferation-inducing growth factor, a multipotent neural stem cell begins to divide giving rise to a cluster of undifferentiated cells referred to herein as a "neurosphere". The cells of a single neurosphere are clonal in nature because they are the progeny of a single neural stem cell. In the continued presence of a proliferation-inducing growth factor such as EGF or the like, precursor cells within the neurosphere continue to divide resulting in an increase in the size of the neurosphere and the number of undifferentiated cells. The neurosphere is not immunoreactive for GFAP, neurofilament (NF), neuron-specific enolase (NSE) or myelin basic protein (MBP). However, precursor cells within the neurosphere are immunoreactive for nestin, an intermediate filament protein found in many types of undifferentiated CNS cells. The nestin marker was characterized by Lehndahl et al., *Cell* 60:585–595 (1990). Antibodies are available to identify nestin, including the rat antibody referred to as Rat401. The mature phenotypes associated with the differentiated cell types that may be derived from the neural stem cell progeny are predominantly negative for the nestin phenotype.

After about 4 to 5 days in the absence of a substrate, the proliferating neurospheres lift off the floor of the culture dish and tend to form the free-floating clusters characteristic of neurospheres. Floating neurospheres are depicted in FIG. 2*d*. It is possible to vary the culture conditions so that while the precursor cells still express the nestin phenotype, they do not form the characteristic neurospheres. The proliferating precursor cells of the neurosphere continue to proliferate in suspension. After about 3–10 days in vitro, and more particularly after about 6–7 days in vitro, the proliferating neurospheres are fed every 2–7 days, preferably every 2–4 days by gentle centrifugation and resuspension in Complete Medium containing a growth factor.

The neurospheres of the suspension culture can be easily passaged to reinitiate proliferation. After 6–7 days in vitro, the culture flasks are shaken well and the neurospheres allowed to settle on the bottom corner of the flask. The neurospheres are then transferred to a 50 ml centrifuge tube and centrifuged at low speed. The medium is aspirated, and the neurospheres are resuspended in a small amount of Complete Medium. Individual cells in the neurospheres can be separated by physical dissociation of the neurospheres with a blunt instrument, for example, by triturating the neurospheres with a pipette, especially a fire polished pasteur pipette, to form a single cell suspension of neural stem cell progeny. The cells are then counted and replated at the desired density to reinitiate proliferation. Single cells from the dissociated neurospheres are suspended in Complete Medium containing growth factor, and a percentage of these cells proliferate and form new neurospheres largely composed of undifferentiated cells. This procedure can be repeated weekly to result in a logarithmic increase in the number of viable cells at each passage. The procedure is continued until the desired number of precursor cells is obtained.

The number of neural stem cell progeny proliferated in vitro from the mammalian CNS can be increased dramatically by injecting a growth factor or combination of growth factors, for example EGF, FGF, or EGF and FGF together, into the ventricles of the donor in vivo using the in vivo proliferation methods described in more detail below. As detailed in Example 31 below, 6 days after infusion of EGF into the lateral ventricle of a mouse forebrain, the walls of the ventricle were removed and the stem cells harvested. Infusion of EGF into the lateral ventricle increased the efficiency of the yield of stem cells that proliferated to form neurospheres.

This ability to enhance the proliferation of neural stem cells should prove invaluable when stem cells are to be harvested for later transplantation back into a patient, thereby making the initial surgery 1) less traumatic because less tissue would have to be removed and 2) more efficient because a greater yield of stem cells per surgery would proliferate in vitro.

Additionally, the patient's stem cells, once they have proliferated in vitro, could also be genetically modified in vitro using the techniques described below. The in vitro genetic modification may be more desirable in certain circumstances than in vivo genetic modification techniques when more control over the infection with the genetic material is required.

Neural stem cell progeny can be cryopreserved until they are needed by any method known in the art. The cells can be suspended in an isotonic solution, preferably a cell culture medium, containing a particular cryopreservant. Such cryopreservants include dimethyl sulfoxide (DMSO), glycerol and the like. These cryopreservants are used at a concentration of 5–15%, preferably 8–10%. Cells are frozen gradually to a temperature of −10° C. to −150° C., preferably −20° C. to −100° C., and more preferably −70° C. to −80° C.

Differentiation of Neural Stem Cell Progeny

Differentiation of the cells can be induced by any method known in the art which activates the cascade of biological events which lead to growth, which include the liberation of inositol triphosphate and intracellular Ca^{2+}, liberation of diacyl glycerol and the activation of protein kinase C and other cellular kinases, and the like. Treatment with phorbol esters, differentiation-inducing growth factors and other chemical signals can induce differentiation. Differentiation can also be induced by plating the cells on a fixed substrate such as flasks, plates, or coverslips coated with an ionically charged surface such as poly-L-lysine and poly-L-ornithine and the like.

Other substrates may be used to induce differentiation such as collagen, fibronectin, laminin, MATRIGEL™ (Collaborative Research), and the like. Differentiation can also be induced by leaving the cells in suspension in the presence of a proliferation-inducing growth factor, without reinitiation of proliferation (i.e. without dissociating the neurospheres).

A preferred method for inducing differentiation of the neural stem cell progeny comprises culturing the cells on a fixed substrate in a culture medium that is free of the proliferation-inducing growth factor. After removal of the proliferation-inducing growth factor, the cells adhere to the

substrate (e.g. poly-ornithine-treated plastic or glass), flatten, and begin to differentiate into neurons and glial cells. At this stage the culture medium may contain serum such as 0.5–1.0% fetal bovine serum (FBS). However, for certain uses, if defined conditions are required, serum would not be used. Within 2–3 days, most or all of the neural stem cell progeny begin to lose immunoreactivity for nestin and begin to express antigens specific for neurons, astrocytes or oligodendrocytes as determined by immunocytochemistry techniques well known in the art.

Immunocytochemistry (e.g. dual-label immunofluorescence and immunoperoxidase methods) utilizes antibodies that detect cell proteins to distinguish the cellular characteristics or phenotypic properties of neurons from astrocytes and oligodendrocytes. In particular, cellular markers for neurons include NSE, NF, β-tub, MAP-2; and for glia, GFAP (an identifier of astrocytes), galactocerebroside (GalC) (a myelin glycolipid identifier of oligodendrocytes), and the like.

Immunocytochemistry can also be used to detect the expression of neurotransmitters, or in some cases the expression of enzymes responsible for neurotransmitter synthesis. For the identification of neurons, antibodies can be used that detect the presence of acetylcholine (ACh), dopamine, epinephrine, norepinephrine, histamine, serotonin or 5-hydroxytryptamine (5-HT), neuropeptides such as substance P, adrenocorticotrophic hormone, vasopressin or antidiuretic hormone, oxytocin, somatostatin, angiotensin II, neurotensin, and bombesin, hypothalamic releasing hormones such as TRH and luteinizing releasing hormone, gastrointestinal peptides such as vasoactive intestinal peptide (VIP) and cholecystokinin (CCK) and CCK-like peptide, opioid peptides such as endorphins like β-endorphin and enkephalins such as met- and leu-enkephalin, prostaglandins, amino acids such as γ-amino butyric acid (GABA), glycine, glutamate, cysteine, taurine and aspartate and dipeptides such as carnosine. Antibodies to neurotransmitter-synthesizing enzymes can also be used such as glutamic acid decarboxylase (GAD) which is involved in the synthesis of GABA, choline acetyltransferase (ChAT) for ACh synthesis, dopa decarboxylase (DDC) for dopamine, dopamine-β-hydroxylase (DBH) for norepinephrine, and amino acid decarboxylase for 5-HT. Antibodies to enzymes that are involved in the deactivation of neurotransmitters may also be useful such as acetyl cholinesterase (AChE) which deactivates ACh. Antibodies to enzymes involved in the reuptake of neurotransmitters into neuronal terminals such as monoamine oxidase and catechol-o-methyl transferase for dopamine, for 5-HT, and GABA transferase for GABA may also identify neurons. Other markers for neurons include antibodies to neurotransmitter receptors such as the AChE nicotinic and muscarinic receptors, adrenergic receptors α^1, α_2, β^1 and α_2, the dopamine receptor and the like. Cells that contain a high level of melanin, such as those found in the substantia nigra, could be identified using an antibody to melanin.

In situ hybridization histochemistry can also be performed, using cDNA or RNA probes specific for the peptide neurotransmitter or the neurotransmitter synthesizing enzyme mRNAs. These techniques can be combined with immunocytochemical methods to enhance the identification of specific phenotypes. If necessary, the antibodies and molecular probes discussed above can be applied to Western and Northern blot procedures respectively to aid in cell identification.

A preferred method for the identification of neurons uses immunocytochemistry to detect immunoreactivity for NSE, NF, NeuN, and the neuron specific protein, tau-1. Because these markers are highly reliable, they will continue to be useful for the primary identification of neurons, however neurons can also be identified based on their specific neurotransmitter phenotype as previously described.

Type I astrocytes, which are differentiated glial cells that have a flat, protoplasmic/fibroblast-like morphology, are preferably identified by their immunoreactivity for GFAP but not A2B5. Type II astrocytes, which are differentiated glial cells that display a stellate process-bearing morphology, are preferably identified using immunocytochemistry by their phenotype GFAP(+), A2B5(+) phenotype.

Cells that do not express intermediate filaments specific for neurons or for astrocytes, begin to express markers specific for oligodendrocytes in a correct temporal fashion. That is, the cells first become immunoreactive for O4, galactocerebroside (GalC, a myelin glycolipid) and finally, MBP. These cells also possess a characteristic oligodendrocyte morphology.

The present invention provides a method of influencing the relative proportion of these differentiated cell types by the addition of exogenous growth factors during he differentiation stage of the precursor cells. By using dual-label mmunofluorescence and immunoperoxidase methods with various neuronal- and glial-specific antibodies, the effect of the exogenous growth factors on the differentiating cells can be determined.

The biological effects of growth and trophic factors are generally mediated through binding to cell surface receptors. The receptors for a number of these factors have been identified and antibodies and molecular probes for specific receptors are available. Neural stem cells can be analyzed for the presence of growth factor receptors at all stages of differentiation. In many cases, the identification of a particular receptor will define the strategy to use in further differentiating the cells along specific developmental pathways with the addition of exogenous growth or trophic factors.

Exogenous growth factors can be added alone or in various combinations. They can also be added in a temporal sequence (i.e. exposure to a first growth factor influences the expression of a second growth factor receptor, *Neuron* 4:189–201 (1990). Among the growth factors and other molecules that can be used to influence the differentiation of precursor cells in vitro are FGF-1, FGF-2, ciliary neurotrophic factor (CNTF), NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3, neurotrophin 4, interleukins, leukemia inhibitory factor (LIF), cyclic adenosine monophosphate, forskolin, tetanus toxin, high levels of potassium, amphiregulin, TGF-α, TGF-β, insulin-like growth factors, dexamethasone (glucocorticoid hormone), isobutyl 3-methylxanthine, somatostatin, growth hormone, retinoic acid, and PDGF. These and other growth factors and molecules will find use in the present invention.

Genetic Modification of Neural Stem Cell Progeny

Although the precursor cells are non-transformed primary cells, they possess features of a continuous cell line. In the undifferentiated state, in the presence of a proliferation-inducing growth factor such as EGF, the cells continuously divide and are therefore excellent targets for genetic modification. The term "genetic modification" as used herein refers to the stable or transient alteration of the genotype of a precursor cell by intentional introduction of exogenous DNA. DNA may be synthetic, or naturally derived, and may

contain genes, portions of genes, or other useful DNA sequences. The term "genetic modification" as used herein is not meant to include naturally occurring alterations such as that which occurs through natural viral activity, natural genetic recombination, or the like.

Exogenous DNA may be introduced to a precursor cell by viral vectors (retrovirus, modified herpes viral, herpes-viral, adenovirus, adeno-associated virus, and the like) or direct DNA transfection (lipofection, calcium phosphate transfection, DEAE-dextran, electroporation, and the like). The genetically modified cells of the present invention possess the added advantage of having the capacity to fully differentiate to produce neurons or macroglial cells in a reproducible fashion using a number of differentiation protocols.

In another embodiment, the precursor cells are derived from transgenic animals, and thus are in a sense already genetically modified. There are several methods presently used for generating transgenic animals. The technique used most often is direct miicroinjection of DNA into single-celled fertilized eggs. Other techniques include retroviral-mediated transfer, or gene transfer in embryonic stem cells. These techniques and others are detailed by Hogan et al. in *Manipulating the Mouse Embryo, A Laboratory Manual* (Cold Spring Harbor Laboratory Ed., 1986). Use of these transgenic animals has certain advantages including the fact that there is no need to transfect healthy neurospheres. Precursor cells derived from transgenic animals will exhibit stable gene expression. Using transgenic animals, it is possible to breed in new genetic combinations. The transgenic animal may have integrated into its genome any useful gene that is expressed by neural cells. Examples of useful DNA are given below in the discussion of genetically modifying precursor cells.

A significant challenge for cellular transplantation in the CNS is the identification of the donor cells after implantation within the host. A number of strategies have been employed to mark donor cells, including tritiated labels, fluorescent dyes, dextrans, and viral vectors carrying reporter genes. However, these methods suffer from inherent problems of toxicity, stability, or dilution over the long term. The use of neural cells derived from transgenic animals may provide an improved means by which identification of transplanted neural cells can be achieved. A transgenic marking system provides a more stable and efficient method for cell labeling. In this system, promoter elements, for example for GFAP and MBP, can direct the expression of the *E. coli* β-galactosidase reporter gene in transgenic mice. In these systems, cell-specific expression of the reporter gene occurs in astrocytes (GFAP-lacZ) and in oligodendrocytes (MBP-lacZ) in a developmentally-regulated manner. The Rosa26 transgenic mice, described in Example 45, is one example of a transgenic marking system in which all cells ubiquitously express β-galactosidase.

Once propagated, the neurosphere cells are mechanically dissociated into a single cell suspension and plated on petri dishes in a medium where they are allowed to attach overnight. The precursor cells are then genetically modified. If the precursor cells are generated from transgenic animals, then they may or may not be subjected to further genetic modification, depending upon the properties desired of the cells. Any useful genetic modification of the cells is within the scope of the present invention. For example, precursor cells may be modified to produce or increase production of a biologically active substance such as a neurotransmitter or growth factor or the like. The genetic modification is performed either by infection with recombinant retroviruses or transfection using methods known in the art (see Maniatis et al., in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. (1982)). Briefly, the chimeric gene constructs will contain viral, for example retroviral long terminal repeat (LTR), simian virus 40 (SV40), cytomegalovirus (CMV); or mammalian cell-specific promoters such as tyrosine hydroxylase (TH, a marker for dopamine cells), DBH, phenylethanolamine N-methyltransferase (PNMT), ChAT, GFAP, NSE, the NF proteins (NF-L, NF-M, NF-H, and the like) that direct the expression of the structural genes encoding the desired protein. In addition, the vectors will include a drug selection marker, such as the *E. coli* aminoglycoside phosphotransferase gene, which when coinfected with the experimental gene confers resistance to geneticin (G418), a protein synthesis inhibitor.

When the genetic modification is for the production of a biologically active substance, the substance will generally be one that is useful for the treatment of a given CNS disorder. For example, it may be desired to genetically modify cells so they secrete a certain growth factor product. As used herein, the term "growth factor product" refers to a protein, peptide, mitogen, or other molecule having a growth, proliferative, differentiative, or trophic effect. Growth factor products useful in the treatment of CNS disorders include, but are not limited to, NGF, BDNF, the neurotrophins (NT-3, NT-4/NT-5), CNTF, amphiregulin, FGF-1, FGF-2, EGF, TGFα, TGFβs, PDGF, IGFs, and the interleukins.

Cells can also be modified to express a certain growth factor receptor (r) including, but not limited to, p75 low affinity NGFr, CNTFr, the trk family of neurotrophin receptors (trk, trkB, trkC), EGFr, FGFr, and amphiregulin receptors. Cells can be engineered to produce various neurotransmitters or their receptors such as serotonin, L-dopa, dopamine, norepinephrine, epinephrine, tachykinin, substance-P, endorphin, enkephalin, histamine, N-methyl D-aspartate, glycine, glutamate, GABA, ACh, and the like. Useful neurotransmitter-synthesizing genes include TH, DDC, DBH, PNMT, GAD, tryptophan hydroxylase, ChAT, and histidine decarboxylase. Genes that encode for various neuropeptides, which may prove useful in the treatment of CNS disorders, include substance-P, neuropeptide-Y, enkephalin, vasopressin, VIP, glucagon, bombesin, CCK, somatostatin, calcitonin gene-related peptide, and the like.

After successfully transfected/infected cells are selected they can be cloned using limiting dilution in 96 multi-well plates and assayed for the presence of the desired biologically active substance. Clones that express high levels of the desired substance are grown and their numbers expanded in T-flasks. The specific cell line can then be cyropreserved. Multiple clones of genetically modified precursor cells will be obtained. Some may give rise preferentially to neuronal cells, and some to glial cells.

The genetically modified precursor cells can be implanted for cell/gene therapy into the CNS of a recipient in need of the biologically active molecule produced by the genetically modified cells. Transplantation techniques are detailed below.

Alternatively, the genetically modified precursor cells can be subjected to various differentiation protocols in vitro prior to implantation. For example, genetically modified precursor cells may be removed from the culture medium which allows proliferation and differentiated using any of the protocols described above. The protocol used will depend upon the type of genetically modified cell desired.

Once the cells have differentiated, they are again assayed for expression of the desired protein. Cells having the desired phenotype can be isolated and implanted into recipients in need of the protein or biologically active molecule that is expressed by the genetically modified cell.

Transplantation of Neural Stem Cell Progeny Alleviate Disorders of the CNS in Animal Models Caused by Disease or Injury

It is well recognized in the art that transplantation of tissue into the CNS offers the potential for treatment of neurodegenerative disorders and CNS damage due to injury (review: Lindvall, (1991) *Tins* vol. 14(8): 376–383). Transplantation of new cells into the damaged CNS has the potential to repair damaged circuitries and provide neurotransmitters thereby restoring neurological function. However, the absence of suitable cells for transplantation purposes has prevented the full potential of this procedure from being met. "Suitable" cells are cells that meet the following criteria: 1) can be obtained in large numbers; 2) can be proliferated in vitro to allow insertion of genetic material, if necessary; 3) capable of surviving indefinitely but stop growing after transplantation to the brain; 4) are nonimmunogenic, preferably obtained from a patient's own tissue; 5) are able to form normal neural connections and respond to neural physiological signals (Bjorklund (1991) *TINS* Vol. 14(8): 319–322). The progeny of multipotent neural stem cells obtainable from embryonic or adult CNS tissue, which are able to divide indefinitely when maintained in vitro using the culture conditions described herein, meet all of the desirable requirements of cells suitable for neural transplantation purposes and are a particularly suitable cell line as the cells have not been immortalized and are not of tumorigenic origin. The use of multipotent neural stem cells in the treatment of neurological disorders and CNS damage can be demonstrated by the use of animal models.

The neural stem cell progeny can be administered to any animal with abnormal neurological or neurodegenerative symptoms obtained in any manner, including those obtained as a result of mechanical, chemical, or electrolytic lesions, as a result of experimental aspiration of neural areas, or as a result of aging processes. Particularly preferable lesions in non-human animal models are obtained with 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), ibotenic acid and the like.

The instant invention allows the use of precursor cells prepared from donor tissue which is xenogeneic to the host. Since the CNS is a somewhat immunoprivileged site, the immune response is significantly less to xenografts, than elsewhere in the body. In general, however, in order for xenografts to be successful it is preferred that some method of reducing or eliminating the immune response to the implanted tissue be employed. Thus recipients will often be immunosuppressed, either through the use of immunosuppressive drugs such as cyclosporin, or through local immunosuppression strategies employing locally applied immunosuppressants. Local immunosuppression is disclosed by Gruber, *Transplantation* 54:1–11 (1992). Rossini, U.S. Pat. No. 5,026,365, discloses encapsulation methods suitable for local immunosuppression.

As an alternative to employing immunosuppression techniques, methods of gene replacement or knockout using homologous recombination in embryonic stem cells, taught by Smithies et al. (*Nature*, 317:230–234 (1985), and extended to gene replacement or knockout in cell lines (H. Zheng 35 al., *PNAS*, 88:8067–8071 (1991)), can be applied to precursor cells for the ablation of major histocompatibility complex (MHC) genes. Precursor cells lacking MHC expression would allow for the grafting of enriched neural cell populations across allogeneic, and perhaps even xenogeneic, histocompatibility barriers without the need to immunosuppress the recipient. General reviews and citations for the use of recombinant methods to reduce antigenicity of donor cells are also disclosed by Gruber (supra). Exemplary approaches to the reduction of immunogenicity of transplants by surface modification are disclosed by Faustman WO 92/04033 (1992). Alternatively the immunogenicity of the graft may be reduced by preparing precursor cells from a transgenic animal that has altered or deleted MHC antigens.

Grafting of precursor cells prepared from tissue which is allogeneic to that of the recipient will most often employ tissue typing in an effort to most closely match the histocompatibility type of the recipient. Donor cell age as well as age of the recipient have been demonstrated to be important factors in improving the probability of neuronal graft survival. The efficiency of grafting is reduced with increased age of donor cells. Furthermore, grafts are more readily accepted by younger recipients compared to older recipients. These two factors are likely to be as important for glial graft survival as they are for neuronal graft survival.

In some instances, it may be possible to prepare neural stem cell progeny from the recipient's own nervous system (e.g.in the case of tumor removal biopsies etc,).

In such instances the neural stem cell progeny may be generated from dissociated tissue and proliferated in vitro using the methods described above. Upon suitable expansion of cell numbers, the precursor cells may be harvested, genetically modified if necessary, and readied for direct injection into the recipient's CNS.

Transplantation can be done bilaterally, or, in the case of a patient suffering from Parkinson's Disease, contralateral to the most affected side. Surgery is performed in a manner in which particular brain regions may be located, such as in relation to skull sutures, particularly with a stereotaxic guide. Cells are delivered throughout any affected neural area, in particular to the basal ganglia, and preferably to the caudate and putamen, the nucleus basalis or the substantia nigra. Cells are administered to the particular region using any method which maintains the integrity of surrounding areas of the brain, preferably by injection cannula. Injection methods exemplified by those used by Duncan et al. *J.Neurocytology*, 17:351–361 (1988), and scaled up and modified for use in humans are preferred. Methods taught by Gage et al., supra, for the injection of cell suspensions such as fibroblasts into the CNS may also be employed for injection of neural precursor cells. Additional approaches and methods may be found in *Neural Grafting in the Mammalian CNS*, Bjorklund and Stenevi, eds., (1985).

Although solid tissue fragments and cell suspensions of neural tissue are immunogenic as a whole, it could be possible that individual cell types within the graft are themselves immunogenic to a lesser degree. For example, Bartlett et al. (*Prog. Brain Res.* 82: 153–160 (1990)) have abrogated neural allograft rejection by pre-selecting a subpopulation of embryonic neuroepithelial cells for grafting by the use of immunobead separation on the basis of MHC expression. Thus, another approach is provided to reduce the chances of allo and xenograft rejection by the recipient without the use of immunosuppression techniques.

Neural stem cell progeny when administered to the particular neural region preferably form a neural graft, wherein

the neuronal cells form normal neuronal or synaptic connections with neighboring neurons, and maintain contact with transplanted or existing glial cells which may form myelin sheaths around the neurons' axons, and provide a trophic influence for the neurons. As these transplanted cells form connections. they re-establish the neuronal networks which have been damaged due to disease and aging.

Survival of the graft in the living host can be examined using various non-invasive scans such as computerized axial tomography (CAT scan or CT scan), nuclear magnetic resonance or magnetic resonance imaging (NMR or MRI) or more preferably positron emission tomography (PET) scans. Post-mortem examination of graft survival can be done by removing the neural tissue, and examining the affected region macroscopically, or more preferably using microscopy. Cells can be stained with any stains visible under light or electron microscopic conditions, more particularly with stains which are specific for neurons and glia. Particularly useful are monoclonal antibodies which identify neuronal cell surface markers such as the M6 antibody which identifies mouse neurons. Most preferable are antibodies which identify any neurotransmitters, particularly those directed to GABA, TH, ChAT, and substance P, and to enzymes involved in the synthesis of neurotransmitters, in particular, GAD. Transplanted cells can also be identified by prior incorporation of tracer dyes such as rhodamine- or fluorescein-labelled microspheres, fast blue, bisbenzamide or retrovirally introduced histochemical markers such as the lac Z gene which produces beta galactosidase.

Functional integration of the graft into the host's neural tissue can be assessed by examining the effectiveness of grafts on restoring various functions, including but not limited to tests for endocrine, motor, cognitive and sensory functions. Motor tests which can be used include those which quantitate rotational movement away from the degenerated side of the brain, and those which quantitate slowness of movement, balance, coordination, akinesia or lack of movement, rigidity and tremors. Cognitive tests include various tests of ability to perform everyday tasks, as well as various memory tests, including maze performance.

Neural stem cell progeny can be produced and transplanted using the above procedures to treat demyelination diseases. Human demyelinating diseases for which the cells of the present invention may provide treatment include disseminated perivenous encephalomyelitis, MS (Charcot and Marburg types), neuromyelitis optica, concentric sclerosis, acute, disseminated encephalomyelitides, post encephalomyelitis, postvaccinal encephalomyelitis, acute hemorrhagic leukoencephalopathy, progressive multifocal leukoencephalopathy, idiopathic polyneuritis, diphtheric neuropathy, Pelizaeus-Merzbacher disease, neuromyelitis optica, diffuse cerebral sclerosis, central pontine myelinosis, spongiform leukodystrophy, and leukodystrophy (Alexander type).

Areas of demyelination in humans is generally associated with plaque like structures. Plaques can be visualized by magnetic resonance imaging. Accessible plaques are the target area for injection of neural stem cell progeny. Standard stereotactic neurosurgical methods are used to inject cell suspensions both into the brain and spinal cord. Generally, the cells can be obtained from any of the sources discussed above. However, in the case of demyelinating diseases with a genetic basis directly affecting the ability of the myelin forming cell to myelinate axons, allogeneic tissue would be a preferred source of the cells as autologous tissue (i.e. the recipient's cells) would generally not be useful unless the cells have been modified in some way to insure the lesion will not continue (e.g. genetically modifying the cells to cure the demyelination lesion).

Oligodendrocytes derived from neural stem cell progeny proliferated and differentiated in vitro may be injected into demyelinated target areas in the recipient. Appropriate amounts of type I astrocytes may also be injected. Type I astrocytes are known to secrete PDGF which promotes both migration and cell division of oligodendrocytes. [Nobel et al., *Nature* 333:560–652 (1988); Richardson et al., *Cell*, 53:309–319 (1988)].

A preferred treatment of demyelination disease uses undifferentiated neural stem cell progeny. Neurospheres grown in the presence of a proliferation-inducing growth factor such as EGF can be dissociated to obtain individual precursor cells which are then placed in injection medium and injected directly into the demyelinated target region. The cells differentiate in vivo. Astrocytes can promote remyelination in various paradigms. Therefore, in instances where oligodendrocyte proliferation is important, the ability of precursor cells to give rise to type I astrocytes may be useful. In other situations, PDGF may be applied topically during the transplantation as well as with repeated doses to the implant site thereafter.

The injection of neural stem cell progeny in remyelination therapy provides, amongst other types of cells, a source of immature type I astrocytes at the implant site. This is a significant feature because immature astrocytes (as opposed to mature astrocytes) have a number of specific characteristics that make them particularly suited for remyelination therapy. First, immature, as opposed to mature, type I astrocytes are known to migrate away from the implant site [Lindsay et. al, *Neurosci.* 12:513–530 (1984)] when implanted into a mature recipient and become associated with blood vessels in the recipient's CNS [Silver et al., WO 91/06631 (1991)]. This is at least partially due to the fact that immature astrocytes are intrinsically more motile than mature astrocytes. [Duffy et al., *Exp Cell Res.* 139:145–157 (1982), Table VII]. Type I astrocytes differentiating at or near the precursor cell implant site should have maximal motility and thereby optimize the opportunity for oligodendrocyte growth and division at sites distant from the implant. The localization of the astrocytes near blood vessels is also significant from a therapeutic standpoint since (at least in MS) most plaques have a close anatomical relationship with one or more veins.

Another characteristic of immature astrocytes that makes them particularly suited for remyelination therapy is that they undergo a lesser degree of cell death than mature type I astrocytes. (Silver et al., supra)

Any suitable method for the implantation of precursor cells near to the demyelinated targets may be used so that the cells can become associated with the demyelinated axons. Glial cells are motile and are known to migrate to, along, and across their neuronal targets thereby allowing the spacing of injections. Remyelination by the injection of precursor cells is a useful therapeutic in a wide range of demyelinating conditions. It should also be borne in mind that in some circumstances remyelination by precursor cells will not result in permanent remyelination, and repeated injections will be required. Such therapeutic approaches offer advantage over leaving the condition untreated and may spare the recipient's life.

In Vivo Proliferation, Differentiation, and Genetic Modification of Neural Stem Cell Progeny

Neural stem cells and their progeny can be induced to proliferate and differentiate in vivo by administering to the

host, any growth factor(s) or pharmaceutical composition that will induce proliferation and differentiation of the cells. These growth factors include any growth factor known in the art, including the growth factors described above for in vitro proliferation and differentiation. Pharmaceutical compositions include any substance that blocks the inhibitory influence and/or stimulates neural stem cells and stem cell progeny to proliferate and ultimately differentiate. Thus, the techniques described above to proliferate, differentiate, and genetically modify neural stem cells in vitro can be adapted to in vivo techniques, to achieve similar results. Such in vivo manipulation and modification of these cells allows cells lost, due to injury or disease, to be endogenously replaced, thus obviating the need for transplanting foreign cells into a patient. Additionally, the cells can be modified or genetically engineered in vivo so that they express various biological agents useful in the treatment of neurological disorders. Administration of growth factors can be done by any method, including injection cannula, transfection of cells with growth hormone-expressing vectors, injection, timed-release apparati which can administer substances at the desired site, and the like. Pharmaceutical compositions can be administered by any method, including injection cannula, injection, oral administration, timed-release apparati and the like. The neural stem cells can be induced to proliferate and differentiate in vivo by induction with particular growth factors or pharmaceutical compositions which will induce their proliferation and differentiation. Therefore, this latter method circumvents the problems associated with transplantation and immune reactions to foreign cells. Any growth factor can be used, particularly EGF, TGFα, FGF-1, FGF-2 and NGF.

Growth factors can be administered in any manner known in the art in which the factors may either pass through or by-pass the blood-brain barrier. Methods for allowing factors to pass through the blood-brain barrier include minimizing the size of the factor, or providing hydrophobic factors which may pass through more easily.

The fact that neural stem cells are located in the tissues lining ventricles of mature brains offers several advantages for the modification and manipulation of these cells in vivo and the ultimate treatment of various neurological diseases, disorders, and injury that affect different regions of the CNS. Therapy for these can be tailored accordingly so that stem cells surrounding ventricles near the affected region would be manipulated or modified in vivo using the methods described herein. The ventricular system is found in nearly all brain regions and thus allows easier access to the affected areas. If one wants to modify the stem cells in vivo by exposing them to a composition comprising a growth factor or a viral vector, it is relatively easy to implant a device that administers the composition to the ventricle and thus, to the neural stem cells. For example, a cannula attached to an osmotic pump may be used to deliver the composition. Alternatively, the composition may be injected directly into the ventricles. The neural stem cell progeny can migrate into regions that have been damaged as a result of injury or disease. Furthermore, the close proximity of the ventricles to many brain regions would allow for the diffusion of a secreted neurological agent by the stem cells or their progeny.

For treatment of Huntington's Disease, Alzheimer's Disease, Parkinson's Disease, and other neurological disorders affecting primarily the forebrain, growth factors or other neurological agents would be delivered to the ventricles of the forebrain to affect in vivo modification or manipulation of the stem cells. For example, Parkinson's Disease is the result of low levels of dopamine in the brain, particularly the striatum. It would be advantageous to induce a patient's own quiescent stem cells to begin to divide in vivo and to induce the progeny of these cells to differentiate into dopaminergic cells in the affected region of the striatum, thus locally raising the levels of dopamine.

Normally the cell bodies of dopaminergic neurons are located in the substantia nigra and adjacent regions of the mesencephalon, with the axons projecting to the striatum. Prior art methods for treating Parkinson's disease usually involves the use of the drug L-Dopa, to raise dopamine levels in the striatum. However, there are disadvantages with this treatment including drug tolerance and side effects. Also, embryonic tissues that produce dopamine have been transplanted into the striatum of human Parkinsonian patients with reasonable success. However, the use of large quantities of fetal human tissue required for this procedure raises serious ethical concerns and practical issues.

The methods and compositions of the present invention provide an alternative to the use of drugs and the controversial use of large quantities of embryonic tissue for treatment of Parkinson's disease. Dopamine cells can be generated in the striatum by the administration of a composition comprising growth factors to the lateral ventricle. A particularly preferred composition comprises a combination of EGF, FGF-2, and heparan sulphate. The composition preferably also comprises serum. After administration of this composition, there is a significant increase in the transcription of messenger RNA (mRNA) for TH in the subventricular region of the striatum, an area which normally does not contain dopaminergic cell bodies. These methods and results are described in detail in Example 34. As detailed in

Example 35, the use of dual labeling tissue to show the distribution of BrdU+ and TH+ cells indicates that, in response to the in vivo administration of growth factors, TH+ cell bodies occur in striatal tissue. Many of these newly generated TH+ cells are also BrdU+.

For the treatment of MS and other demyelinating or hypomyelinating disorders, and for the treatment of Amyotrophic Lateral Sclerosis or other motor neuron diseases, growth factors or other neurological agents would be delivered to the central canal.

In addition to treating CNS tissue immediately surrounding a ventricle, a viral vector, DNA, growth factor, or other neurological agent can be easily administered to the lumbar cistern for circulation throughout the CNS.

Under normal conditions subependymal precursors do not differentiate or migrate, rather, their fate appears to be cell death after an undefined number of cell divisions (Morshead and Van der Kooy, supra). This explanation is also supported by PCR evidence, as described above. Injection of growth factors into the lateral ventricle alters this fate. As described in more detail in Example 27 below, retroviruses were injected into the lateral ventricles for six consecutive days. Implanting cannulae attached to EGF-filled osmotic pumps into the lateral ventricles on the same day as (and 1 or 6 days following) retrovirus injection results in an increase in the total number of RV-β-gal labelled cells 6 days later (from an average of 20 cells/brain to 150 cells/brain).

It is known from the PCR experiments described above that 6 days following retroviral injection no cells exist that contain non-expressed retroviral DNA. Thus these results indicate that the EGF-induced increase in β-gal positive cell number is due to the expansion of the clone size of the retrovirally labelled constitutively proliferative population. It is also possible that part of this increase is due to the activation by EGF of a relatively quiescent stem cell.

Interestingly, this expansion of the number of β-gal labelled cells is accompanied by the migration of these cells away from the subependymal medially, laterally, rostrally, and caudally with subsequent differentiation. Thus, infusion of EGF or similar growth factors induces the proliferation, migration and differentiation of neural stem cells and progenitor cells in vivo, and can be used therapeutically to replace neural cells lost due to injury or disease. In a preferred embodiment EGF and FGF are administered together or sequentially.

The normal fate of the constitutively proliferating cell population (i.e. cell death) can be altered by administering Bcl-2 or genetically modifying the cells with the bcl-2 gene. The gene product is known to prevent programmed cell death (apoptosis) in a variety of cell types. Similar to the EGF experiments, a clonal expansion of the constitutively proliferating cell population is achieved following infection with bcl-2.

Other ways of passing the blood-brain barrier include in vivo transfection of neural stem cells and stem cell progeny with expression vectors containing genes that code for growth factors, so that the cells themselves produce the factor. Any useful genetic modification of the cells is within the scope of the present invention. For example, in addition to genetic modification of the cells to express growth factors, the cells may be modified to express other types of neurological agents such as neurotransmitters. Preferably, the genetic modification is performed either by infection of the cells lining ventricular regions with recombinant retroviruses or transfection using methods known in the art including $CaPO_4$ transfection, DEAE-dextran transfection, polybrene transfection, by protoplast fusion, electroporation, lipofection, and the like [see Maniatis et al., supra]. Any method of genetic modification, now known or later developed can be used. With direct DNA transfection, cells could be modified by particle bombardment, receptor mediated delivery, and cationic liposomes. When chimeric gene constructs are used, they generally will contain viral, for example retroviral long terminal repeat (LTR), simian virus 40 (SV40), cytomegalovirus (CMV); or mammalian cell-specific promoters such as those for TH, DBH, phenylethanolamine N-methyltransferase, ChAT, GFAP, NSE, the NF proteins (NF-L, NF-M, NF-H, and the like) that direct the expression of the structural genes encoding the desired protein.

If a retroviral construct is to be used to genetically modify normally quiescent stem cells, then it is preferable to induce the proliferation of these cells using the methods described herein. For example, an osmotic infusion pump could be used to deliver growth factors to the central canal several days prior to infection with the retrovirus. This assures that there will be actively dividing neural stem cells which are susceptible to infection with the retrovirus.

When the genetic modification is for the production of a biologically active substance, the substance will generally be one that is useful for the treatment of a given CNS disorder. For example, it may be desired to genetically modify cells so they secrete a certain growth factor product. Growth factor products useful in the treatment of CNS disorders are listed above. Cells can also be modified in vivo to express a growth factor receptors, neurotransmitters or their receptors, neurotransmitter-synthesizing genes, neuropeptides, and the like, as discussed above.

Any expression vector known in the art can be used to express the growth factor, as long as it has a promoter which is active in the cell, and appropriate termination and polyadenylation signals. These expression vectors include recombinant vaccinia virus vectors including pSC11, or vectors derived various viruses such as from Simian Virus 40 (SV40, i.e. pSV2-dhfr, pSV2neo, pko-neo, pSV2gpt, pSVT7 and pBABY), from Rous Sarcoma Virus (RSV, i.e. pRSVneo), from mouse mammary tumor virus (MMTV, i.e. pMSG), from adenovirus(pMT2), from herpes simplex virus (HSV, i.e. pTK2 and pHyg), from bovine papillomavirus (BPV, i.e. pdBPV and pBV-1MTHA), from Epstein-Barr Virus (EBV. i.e. p205 and pHEBo) or any other eukaryotic expression vector known in the art.

Other methods for providing growth factors to the area of transplantation include the implantation into the brain in proximity to the graft of any device which can provide an infusion of the factor to the surrounding cells.

In Vitro Models of CNS Development, Function and Dysfunction, and Methods for Screening Effects of Drugs on Neural Cells

Neural stem cell progeny cultured in vitro can be used for the screening of potential neurologically therapeutic compositions. These compositions can be applied to cells in culture at varying dosages, and the response of the cells monitored for various time periods. Physical characteristics of the cells can be analyzed by observing cell and neurite growth with microscopy. The induction of expression of new or increased levels of proteins such as enzymes, receptors and other cell surface molecules, or of neurotransmitters, amino acids, neuropeptides and biogenic amines can be analyzed with any technique known in the art which can identify the alteration of the level of such molecules. These techniques include immunohistochemistry using antibodies against such molecules, or biochemical analysis. Such biochemical analysis includes protein assays, enzymatic assays, receptor binding assays, enzyme-linked immunosorbant assays (ELISA), electrophoretic analysis, analysis with high performance liquid chromatography (HPLC), Western blots, and radioimmune assays (RIA). Nucleic acid analysis such as Northern blots can be used to examine the levels of mRNA coding for these molecules, or for enzymes which synthesize these molecules.

Alternatively, cells treated with these pharmaceutical compositions can be transplanted into an animal, and their survival, ability to form neuronal connections, and biochemical and immunological characteristics examined as previously described.

For the preparation of CNS models, neural stem cells and stem cell progeny are proliferated using the methods described above. Upon removal of the proliferation-inducing growth factor, proliferation of multipotent neural stem cells ceases. The neurospheres can be differentiated using the methods described above, for example by adhering the neurospheres to a substrate such as poly-ornithinetreated plastic or glass where the precursor cells begin to differentiate into neurons and glial cells. Thus, the proliferation-inducing growth factor acts as an extrinsic signaling molecule that can be added or removed at will to control the extent of proliferation.

When the proliferation-inducing growth factor is removed, the growth-factor responsive stem cell progeny can be co-cultured on a feeder layer. Many types of feeder layers may be used, such as fibroblasts, neurons, astrocytes, oligodendrocytes, tumor cell lines, genetically altered cell lines or any cells or substrate with bioactive properties. The feeder layer generally produces a broader range of phenotypes. In this instance, the feeder layer acts as a substrate and

source of both membrane bound and soluble factors that induce and alter the differentiation of the stem cell-generated progeny. Compared to a more inert substance, such as poly-L-ornithine, an astrocyte feeder layer, for example, induces a broader range of neuronal phenotypes as determined by indirect immunocytochemistry at 7 DIV. When differentiated on a poly-L-ornithine coated substrate with 1% FBS, neuronal phenotypes are almost exclusively GABAergic or substance P-ergic. When differentiated on an astrocyte feeder layer, in addition to GABAergic and substance P-ergic neurons, somatostatin, neuropeptide Y (NPY), glutamate and met-enkephalin-containing neurons are present. The astrocytes can be derived from tissue obtained from various brain regions such as the striatum, cortex and spinal cord.

Once the growth factor is removed, the culture medium may contain serum such as 0.5–1.0% FBS. Serum tends to support the differentiation process and enhance cell survival, especially when the differentiating cells are grown at a low density. However, it is possible to culture and differentiate the cells using defined conditions.

Within 1–3 days after removal of the growth factor and placing of the cell in conditions that support differentiation and survival, most or all of the precursor cells begin to lose immunoreactivity for nestin and begin to express antigens specific for neurons, astrocytes or oligodendrocytes. The identification of neurons is confirmed using immunoreactivity for the neuron-specific markers previously mentioned.

The precursor cells described above can be used in methods of determining the effect of a biological agents on neural cells. The term "biological agent" refers to any agent, such as a virus, protein, peptide, amino acid, lipid, carbohydrate, nucleic acid, nucleotide, drug, pro-drug or other substance that may have an effect on neural cells whether such effect is harmful, beneficial, or otherwise. Biological agents that are beneficial to neural cells are referred to herein as "neurological agents", a term which encompasses any biologically or pharmaceutically active substance that may prove potentially useful for the proliferation, differentiation or functioning of CNS cells or treatment of neurological disease or disorder. For example, the term may encompass certain neurotransmitters, neurotransmitter receptors, growth factors, growth factor receptors, and the like, as well as enzymes used in the synthesis of these agents.

Examples of biological agents include growth factors such as FGF-1, FGF-2, EGF and EGF-like ligands, TGFα, IGF-1, NGF, PDGF, and TGFβs; trophic factors such as BDNF, CNTF, and glial-derived neurotrophic factor (GDNF); regulators of intracellular pathways associated with growth factor activity such as phorbol 12-myristate 13-acetate, staurosporine, CGP-41251, tyrphostin, and the like; hormones such as activin and TRH; various proteins and polypeptides such as interleukins, the Bcl-2 gene product, bone morphogenic protein (BMP-2), macrophage inflammatory proteins (MIP-1α, MIP-1β and MIP-2); oligonucleotides such as antisense strands directed, for example, against transcripts for EGF receptors, FGF receptors, and the like; heparin-like molecules such as heparan sulfate; and a variety of other molecules that have an effect on neural stem cells or stem cell progeny including amphiregulin, retinoic acid, and tumor necrosis factor alpha (TNFα).

To determine the effect of a potential biological agent on neural cells, a culture of precursor cells derived from multipotent stem cells can be obtained from normal neural tissue or, alternatively, from a host afflicted with a CNS disease or disorder such as Alzheimer's Disease, Parkinson's Disease, or Down's Syndrome. The choice of culture will depend upon the particular agent being tested and the effects one wishes to achieve. Once the cells are obtained from the desired donor tissue, they are proliferated in vitro in the presence of a proliferation-inducing growth factor.

The ability of various biological agents to increase, decrease or modify in some other way the number and nature of the stem cell progeny proliferated in the presence of EGF or other proliferative factor can be screened on cells proliferated by the methods described in Examples 1–6. For example, it is possible to screen for biological agents that increase the proliferative ability of progenitor cells which would be useful for generating large numbers of cells for transplantation purposes. It is also possible to screen for biological agents which inhibit precursor cell proliferation. In these studies precursor cells are plated in the presence of the biological factor(s) of interest and assayed for the degree of proliferation which occurs. The effects of a biological agent or combination of biological agents on the differentiation and survival of progenitor cells and their progeny can be determined. It is possible to screen neural cells which have already been induced to differentiate prior to the screening. It is also possible to determine the effects of the biological agents on the differentiation process by applying them to precursor cells prior to differentiation. Generally, the biological agent will be solubilized and added to the culture medium at varying concentrations to determine the effect of the agent at each dose. The culture medium may be replenished with the biological agent every couple of days in amounts so as to keep the concentration of the agent somewhat constant.

Changes in proliferation are observed by an increase or decrease in the number of neurospheres that form and/or an increase or decrease in the size of the neurospheres (which is a reflection of the rate of proliferation—determined by the numbers of precursor cells per neurosphere). Thus, the term "regulatory factor" is used herein to refer to a biological factor that has a regulatory effect on the proliferation of stem cells and/or precursor cells. For example, a biological factor would be considered a "regulatory factor" if it increases or decreases the number of stem cells that proliferate in vitro in response to a proliferation-inducing growth factor (such as EGF). Alternatively, the number of stem cells that respond to proliferation-inducing factors may remain the same, but addition of the regulatory factor affects the rate at which the stem cell and stem cell progeny proliferate. A proliferative factor may act as a regulatory factor when used in combination with another proliferative factor. For example, the neurospheres that form in the presence of a combination of bFGF and EGF are significantly larger than the neurospheres that form in the presence of bFGF alone, indicating that the rate of proliferation of stem cells and stem cell progeny is higher.

Other examples of regulatory factors include heparan sulfate, TGFBs, activin, BMP-2, CNTF, retinoic acid, TNFα, MIP-1α, MIP-1β, MIP-2, NGF, PDGF, interleukins, and the Bcl-2 gene product. Antisense molecules that bind to transcripts of proliferative factors and the transcripts for their receptors also regulate stem cell proliferation. Other factors having a regulatory effect on stem cell proliferation include those that interfere with the activation of the c-fos pathway (an intermediate early gene, known to be activated by EGF), including phorbol 12 myristate 13-acetate (PMA; Sigma), which up-regulates the c-fos pathway and staurosporine (Research Biochemical International) and CGP-

41251 (Ciba-Geigy), which down regulate c-fos expression and factors, such as tyrphostin [Fallon, D et al., *Mol. Cell Biol.*, 11 (5): 2697–2703 (1991)] and the like, which suppress tyrosine kinase activation induced by the binding of EGF to its receptor.

Preferred regulatory factors for increasing the rate at which neural stem cell progeny proliferate in response to FGF are heparan sulfate and EGF. Preferred regulatory factors for decreasing the number of stem cells that respond to proliferative factors are members of the TGFβ family, interleukins, MIPs, PDGF, TNFα, retinoic acid (10^{-6}M) and CNTF. Preferred factors for decreasing the size of neurospheres generated by the proliferative factors are members of the TGFβ family, retinoic acid (10^{-6}M) and CNTF.

The regulatory factors are added to the culture medium at a concentration in the range of about 10 pg/ml to 500 ng/ml, preferably about 1 ng/ml to 100 ng/ml. The most preferred concentration for regulatory factors is about 10 ng/ml. The regulatory factor retinoic acid is prepared from a 1 mM stock solution and used at a final concentration between about 0.01 μM and 100 μM, preferably between about 0.05 to 5 μM. Preferred for reducing the proliferative effects of EGF or bFGF on neurosphere generation is a concentration of about 1 μM of retinoic acid. Antisense strands, can be used at concentrations from about 1 to 25 μM. Preferred is a range of about 2 to about 7 μM. PMA and related molecules, used to increase proliferation, may be used at a concentration of about 1 μg/ml to 500 μg/ml, preferably at a concentration of about 10 μg/ml to 200 μg/ml. The glycosaminoglycan, heparan sulfate, is a ubiquitous component on the surface of mammalian cells known to affect a variety of cellular processes, and which binds to growth factor molecules such as FGF and amphiregulin, thereby promoting the binding of these molecules to their receptors on the surfaces of cells. It can be added to the culture medium in combination with other biological factors, at a concentration of about 1 ng/ml to 1 mg/ml; more preferred is a concentration of about 0.2 μg/ml to 20 μg/ml, most preferred is a concentration of about 2 μg/ml.

Using these screening methods, it is possible to screen for potential drug side-effects on pre- and post-natal CNS cells by testing for the effects of the biological agents on stem cell and progenitor cell proliferation and on progenitor cell differentiation or the survival and function of differentiated CNS cells. The proliferated precursor cells are typically plated at a density of about $5–10\times10^6$ cells/ml. If it is desired to test the effect of the biological agent on a particular differentiated cell type or a given make-up of cells, the ratio of neurons to glial cells obtained after differentiation can be manipulated by separating the different types of cells. For example, the O4 antibody (available from Boerhinger Mannheim) binds to oligodendrocytes and their precursors. Using a panning procedure, oligodendrocytes are separated out. Astrocytes can be panned out after a binding procedure using the RAN 2 antibody (available from ATCC). Tetanus toxin (available from Boerhinger Mannheim) can be used to select out neurons. By varying the trophic factors added to the culture medium used during differentiation it is possible to intentionally alter the phenotype ratios. Such trophic factors include EGF, FGF, BDNF, CNTF, TGFα, GDNF, and the like. For example, FGF increases the ratio of neurons, and CNTF increases the ratio of oligodendrocytes. Growing the cultures on beds of glial cells obtained from different CNS regions will also affect the course of differentiation as described above. The differentiated cultures remain viable (with phenotype intact) for at least a month.

The effects of the biological agents are identified on the basis of significant difference relative to control cultures with respect to criteria such as the ratios of expressed phenotypes (neurons: glial cells, or neurotransmitters or other markers), cell viability and alterations in gene expression. Physical characteristics of the cells can be analyzed by observing cell and neurite morphology and growth with microscopy. The induction of expression of new or increased levels of proteins such as enzymes, receptors and other cell surface molecules, or of neurotransmitters, amino acids, neuropeptides and biogenic amines can be analyzed with any technique known in the art which can identify the alteration of the level of such molecules. These techniques include immunohistochemistry using antibodies against such molecules, or biochemical analysis. Such biochemical analysis includes protein assays, enzymatic assays, receptor binding assays, enzyme-linked immunosorbant assays (ELISA), electrophoretic analysis, analysis with high performance liquid chromatography (HPLC), Western blots, and radioimmune assays (RIA). Nucleic acid analysis such as Northern blots and PCR can be used to examine the levels of mRNA coding for these molecules, or for enzymes which synthesize these molecules.

The factors involved in the proliferation of stem cells and the proliferation, differentiation and survival of stem cell progeny, and/or their responses to biological agents can be isolated by constructing cDNA libraries from stem cells or stem cell progeny at different stages of their development using techniques known in the art. The libraries from cells at one developmental stage are compared with those of cells at different stages of development to determine the sequence of gene expression during development and to reveal the effects of various biological agents or to reveal new biological agents that alter gene expression in CNS cells. When the libraries are prepared from dysfunctional tissue, genetic factors may be identified that play a role in the cause of dysfunction by comparing the libraries from the dysfunctional tissue with those from normal tissue. This information can be used in the design of therapies to treat the disorders. Additionally, probes can be identified for use in the diagnosis of various genetic disorders or for use in identifying neural cells at a particular stage in development.

Electrophysiological analysis can be used to determine the effects of biological agents on neuronal characteristics such as resting membrane potential, evoked potentials, direction and ionic nature of current flow and the dynamics of ion channels. These measurements can be made using any technique known in the art, including extracellular single unit voltage recording, intracellular voltage recording, voltage clamping and patch clamping. Voltage sensitive dyes and ion sensitive electrodes may also be used.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the scope of the invention, as defined by the appended claims.

EXAMPLE 1: Dissociation of Embryonic Neural Tissue

14-day-old CD_7 albino mouse embryos (Charles River) were decapitated and the brain and striata were removed using sterile procedure. Tissue was mechanically dissociated with a fire-polished Pasteur pipette into serum-free medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient (Gibco). Dissociated cells were centrifuged at 800 r.p.m. for 5 minutes, the supernatant aspirated, and the cells resuspended in DMEM/F-12 medium for counting.

EXAMPLE 2: Dissociation of Adult Neural Tissue

Brain tissue from juvenile and adult mouse brain tissue was removed and dissected into 500 μm sections and immediately transferred into low calcium oxygenated artificial cerebrospinal fluid (low Ca^{2}+ aCSF) containing 1.33 mg/ml trypsin, 0.67 mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid. Tissue was stirred in this solution for 90 minutes at 32° C.–35° C. aCSF was poured off and replaced with fresh oxygenated aCSF for 5 minutes. Tissue was transferred to DMEM/F-12/10% hormone solution containing 0.7 mg/ml ovomucoid and triturated with a fire polished pasteur pipette. Cells were centrifuged at 400 rpm. for 5 minutes, the supernatant aspirated and the pelleted cells resuspended in DMEM/F-12/10% hormone mix.

EXAMPLE 3: Proliferation of Neural Stem Cells on Substrates

2500 cells/cm^{2} prepared as in Example 1 were plated on poly-L-ornithine-coated (15 μg/ml;Siqma) glass coverslips in 24 well Nunclon (0.5 ml/well) culture dishes. The culture medium was a serum-free medium composed of DMEM/F-12 (1:1) including glucose (0.6%), glutamine (2 gM), sodium bicarbonate (3 mM), and HEPES (4-[2hydroxyethyl]-1-piperazineethanesulfonic acid) buffer (5 mM) (all from Sigma except glutamine [Gibco]). A defined hormone mix and salt mixture (Sigma) that included insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and selenium chloride (30 nM) was used in place of serum. Cultures contained the above medium, hereinafter referred to as "Complete Medium" together with 16–20 ng/ml EGF (purified from mouse sub-maxillary, Collaborative Research) or TGFα (human recombinant, Gibco). After 10–14 days in vitro, media (DMEM only plus hormone mixture) and growth factors were replaced. This medium change was repeated every two to four days. The number of surviving cells at 5 days in vitro was determined by incubating the coverslips in 0.4% trypan blue (Gibco) for two minutes, washing with phosphate buffered saline (PBS, pH 7.3) and counting the number of cells that excluded dye with a Nikon Diaphot inverted microscope.

EXAMPLE 4: Proliferation of Embryonic Mouse Neural Stem Cells in Suspension

Dissociated mouse brain cells prepared as in Examples 1 and 2 (at 1×10^{5} cell/ml) were suspended in Complete Medium with 20 ng/ml of EGF or TGFα. Cells were seeded in a T25 culture flask and housed in an incubator at 37° C., 100% humidity, 95% air/5% CO_2. Cells began to proliferate within 3–4 days and due to a lack of substrate lifted off the floor of the flask and continued to proliferate in suspension forming clusters of undifferentiated cells, referred to herein as "neurospheres". After 6–7 days in vitro the proliferating clusters (neurospheres) were fed every 2–4 days by gentle centrifugation and resuspension in DMEM with the additives described above.

EXAMPLE 5: Proliferation of Adult Mouse Neural Stem Cells in Suspension

The striata, including the subependymal region, of female, pathogen-free CD1 albino mice [3 to 18 month old; Charles River (CF1 and CF2 strains yielded identical results)] were dissected and hand cut with scissors into 1-mm coronal sections and transferred into aCSF (pH 7.35, approx. 180 mOsmol), aerated with 95% O_2–5% CO_2 at room temperature. After 15 minutes the tissue sections were transferred to a spinner flask (Bellco Glass) with a magnetic stirrer filled with low-Ca^{2}+ aCSF (pH 7.35, approx. 180 mOsmol), aerated with 95% O_2–5% CO_2 at 32° to 35° C., containing 1.33 mg/ml of trypsin (9000 BAEE units/mg), 0.67 mg/ml of hyaluronidase (2000 units/mg) and 0.2 mg/ml of kynurenic acid. After 90 minutes, tissue sections were transferred to normal aCSF for 5 minutes prior to trituration. Tissue was transferred to DMEM/F-12 (A: 1, Gibco) medium containing 0.7 mg/ml ovomucoid (Sigma) and triturated mechanically with a fire-narrowed pasteur pipet. Cells were plated (1000 viable cells per plate) in noncoated 35 mm culture dishes (Costar) containing Complete Medium and EGF [20 ng/ml, purified from mouse sub-maxillary gland (Collaborative Research)] or human recombinant (Gibco/BRL). Cells were allowed to settle for 3–10 minutes after which the medium was aspirated away and fresh DMEM/F-12/hormone mix/EGF was added. After 5–10 days in vitro the number of spheres (neurospheres) were counted in each 35 mm dish.

EXAMPLE 6: Passaging Proliferated Stem Cells

After 6–7 days in vitro, individual cells in the neurospheres from Example 4 were separated by triturating the neurospheres with a fire polished pasteur pipette. Single cells from the dissociated neurospheres were suspended in tissue culture flasks in DMEM/F-12/10% hormone mix together with 20 ng/ml of EGF. A percentage of dissociated cells began to proliferate and formed new neurospheres largely composed of undifferentiated cells. The flasks were shaken well and neurospheres were allowed to settle in the bottom corner of the flask. The neurospheres were then transferred to 50 ml centrifuge tubes and centrifuged at 300 rpm for 5 minutes. The medium was aspirated off, and the neurospheres were resuspended in 1 ml of medium containing EGF. The cells were dissociated with a fire-narrowed pasteur pipette and triturated forty times. 20 microliters of cells were removed for counting and added to 20 microliters of Trypan Blue diluted 1:2. The cells were counted and replated at 50,000 cells/ml. This procedure can be repeated weekly and results in a logarithmic increase in the number of viable cells at each passage. The procedure is continued until the desired number of stem cell progeny is obtained.

EXAMPLE 7. Differentiation of Neural Stem Cell Progeny and Immunocytochemistry

Cells proliferated from Examples 4 and 6 were induced to differentiate by maintaining the cells in the culture flasks in the presence of EGF or TGFα at 20 ng/ml without reinitiating proliferation by dissociation of the neurospheres or by plating on poly-ornithine in the continued presence of EGF or TGFα.

Indirect immunocytochemistry was carried out with cells prepared as in Example 3 which had been cultured for 14-30 days in vitro on glass coverslips. For anti-NSE (or anti-nestin) and anti-GFAP immunocytochemistry, cells were fixed with 4% paraformaldehyde in PBS and 95% ethanol/5% acetic acid, respectively. Following a 30 minute fixation period, coverslips were washed three times (10 minutes each) in PBS (pH=7.3) and then incubated in the primary antiserum (NSE 1:300, nestin 1:1500 or GFAP 1:100) in PBS/10% normal goat serum/0.3% TRITON®-X-100) for two hours at 37° C. Coverslips were washed three times (10 minutes each) in PBS and incubated with secondary antibodies (goat-anti-rabbit-rhodamine for anti-NSE or anti-nestin and goat-anti-mouse-fluorescein for antiGFAP, both at 1:50) for 30 minutes at 37° C. Coverslips were then

washed three times (10 minutes each) in PBS, rinsed with water, placed on glass slides and coverslipped using Fluorsave, a mounting medium preferable for use with fluorescein-conjugated antibodies. Fluorescence was detected and photographed with a Nikon Optiphot photomicroscope.

Neural stem cell progeny were also differentiated using the following differentiation paradigms. The neurospheres used for each paradigm were generated as outlined in Examples 4 and 6. All the neurospheres used were passaged at least once prior to their differentiation.

Paradigm 1—Rapid differentiation of neurospheres

Six to 8 days after the first passage, the neurospheres were removed and centrifuged at 400 r.p.m. The EGF-containing supernatant was removed and the pellet suspended in EGF-free complete medium containing 1% FBS. Neurospheres (approximately 0.5–1.0×10^6 cells/well) were plated on poly-L-ornithine-coated (15 μg/ml) glass coverslips in 24 well Nuclon (1.0 ml/well) culture dishes. After 24 hours in culture, the coverslips were transferred to 12 well (Costar) culture dishes containing complete medium containing 0.5% FBS. The medium was changed every 4–7 days. This differentiation procedure is referred to as the "Rapid Differentiation Paradigm" or RDP.

Paradigm 2—Differentiation of dissociated neurospheres

Six to 8 days after the first passage, the neurospheres were removed and centrifuged at 400 r.p.m. The EGF-containing media was removed and the pellet was suspended in EGF-free complete medium containing 1% FBS. The neurospheres were mechanically dissociated into single cells with a fire-polished Pasteur pipette and centrifuged at 800 r.p.m. for 5 minutes. Between 0.5×10^6 and 1.0×10^6 cells were plated on poly-L-ornithine-coated (15 μg/ml) glass coverslips in 24 well Nuclon (1.0 ml/well) culture dishes. The EGF-free culture medium containing 1% FBS was changed every 4–7 days.

Paradigm 3—Differentiation of single neurospheres

Neurospheres were washed free of EGF by serial transfers through changes of EGF-free medium. A single neurosphere was plated onto poly-L-ornithine-coated (15 μg/ml) glass coverslips in a 24-well plate. The culture medium used was complete medium with or without 1% FBS. The medium was changed every 4–7 days.

Paradigm 4—Differentiation of single dissociated neurospheres

Neurospheres were washed free of EGF by serial transfers through changes of EGF-free medium. A single neurosphere was mechanically dissociated in a 0.5 ml Eppendorf centrifuge tube and all the cells were plated onto a 35 mm culture dish. Complete medium was used with or without 1% FBS.

Paradigm 5—Differentiation of neurospheres co-cultured with striatal astrocytes

Neurospheres, derived from striatal cells as described in Example 1 were labeled with 5-bromodeoxyuridine (BrdU) and washed free of EGF. An astrocyte feeder layer was generated from striatal tissue of postnatal mice (0–24 hours), and plated on poly-L-ornithine-coated glass coverslips in a 24-well culture dish. When the astrocytes were confluent, a dissociated or intact neurosphere was placed on each astrocyte bed. Complete medium was changed after the first 24 hours and then every forty-eight hours. When differentiated on an astrocyte feeder layer, in addition to GABAergic and substance P-ergic neurons, somatostatin, NPY, glutamate and methenkephalin-containing neurons were present.

EXAMPLE 8: Effect of Growth Factors on Neurosphere Differentiation

The effects of CNTF, FGF-2, BDNF, and retinoic acid on neurosphere differentiation were tested using the differentiation paradigms set forth in Example 7.

CNTF

The effect of CNTF was assayed in paradigms 1 and 3. For both paradigms, CNTF was added either at the beginning of the experiment at a concentration of 10 ng/ml or daily at a concentration of 1 ng/ml. In paradigm 1, the addition of CNTF increased the number of NSE-immunoreactive cells in addition to the number of tau-1-immunoreactive cells, suggesting that CNTF has an effect on the proliferation, survival, or differentiation of neurons. Preliminary testing with antibodies recognizing the neurotransmitters GABA and substance P suggest that there is no increase in the number of cells containing these proteins. This suggests that a different neuronal phenotype is being produced.

Three different antibodies directed against O4, galactocerebroside (GalC) and MBP were used to study the effect of CNTF on the oligodendrocytes of paradigm 1. CNTF had no effect on the number of O4(+) cells, but there was an increase in the number of GalC(+) and MBP(+) cells compared with the control. Thus it appears that CNTF plays a role in the maturation of oligodendrocytes.

In one experiment, the neurospheres were differentiated as outlined in paradigm 1 except that serum was never added to the culture medium. While the effect of CNTF on neurons and oligodendrocytes was not as apparent as in the presence of serum, there was an increase in the proliferation of flat, protoplasmic astrocytes. Hence, CNTF will affect astrocyte differentiation in various culture conditions.

In paradigm 3, the addition of CNTF resulted in an increase in the number of NSE(+) cells.

BDNF

The effect of BDNF was tested using Paradigm 3. There was an increase in the number of NSE(+) neurons per neurosphere. Additionally, there was an increase in the neuronal branching and the migration of the neurons away from the sphere.

FGF-2

The effect of FGF-2 was tested using paradigms 2 and 4. In paradigm 2, 20 ng/ml of FGF-2 was added at the beginning of the experiment and cells were stained 7 days later. FGF-2 increased the number of GFAP(+) cells and the number of NSE(+) cells. This suggests that FGF-2 has a proliferative or survival effect on the neurons and astrocytes.

In paradigm 4, 20 ng/ml of FGF-2 was added at the beginning of the experiment and assayed 7–10 days later. FGF-2 induced the proliferation of neural stem cell progeny generated by the EGF-responsive stem cell. It induced two different cell types to divide, neuroblasts and bipotential progenitor cells. The neuroblast produced, on average, 6 neurons while the bipotential cell produced approximately 6 neurons and a number of astrocytes.

In previous studies, it was found that when plated at low density (2500 cells/cm^2), addition of EGF up to 7 days in vitro (DIV) could initiate proliferation of the stem cell, but not if applied after 7 DIV. Striatal cells (E14, 2500 cell/cm^2) were plated in the absence or presence of 20 ng/ml of FGF-2. After 11 DIV, cultures were washed and medium containing 20 ng/ml of EGF was added. After 4–5 DIV, in cultures that were primed with FGF-2, greater than 70% of the wells examined contained clusters of proliferating cells that, developed into colonies with the morphologic and antigenic properties of the EGF-generated cells. Cultures that had not been primed with FGF-2 showed no EGF-responsive proliferation. These findings suggest that the EGF-responsive stem cells possess FGF-2 receptors that regulate its long term survival.

Retinoic acid

The effect of retinoic acid at 10^{-7}M was tested using paradigm 1. There was an increase in the number of NSE(+) and tau-1(+) cells, suggesting that retinoic acid increases the number of neurons.

EXAMPLE 9: Proliferation of Embryonic Human Neural Stem Cells and Differentiation of the Neural Stem Cell Progeny

With approval of the Research Ethical Committee at the University of Lund and the Ethics Committee at the University of Calgary, nine 8–12 week old human fetuses were obtained by suction abortions. Tissue was dissected and any identifiable brain regions were removed. Within 4–5 days post-dissection, tissue pieces were mechanically dissociated into single cells using the procedure of Example 1 and the number of viable cells was counted. About 0.1×10^6–0.5×10^6 cells were plated in 35 cm^2 tissue culture flasks (without substrate pretreatment) in Complete Medium with 20 ng/ml of human recombinant EGF (Gibco/BRL).

Two to three days after plating the cells, the majority of the viable cells had extended processes and had taken on a neuronal morphology. By seven days in vitro (DIV), the neuronal-like cells began to die and by 14 DIV nearly all of these cells were dead or dying (determined by the absence of processes, irregular membranes and granular cytoplasm). A few of the cells (1%) did not extend any processes or flatten nor did they take on an astrocytic morphology, instead these cells remained rounded and by 5 to 7 DIV began to divide. By 10 to 14 DIV, small clusters of cells, attached to the substrate, were identified. During the next 7 to 10 days (17 to 24 DIV), these small clusters continued to grow in size and many remained attached to the substrate. By 28 to 30 DIV, nearly all the proliferating clusters had lifted off the substrate and were floating in suspension. While floating in suspension, the clusters continued to grow in size and were passaged after they had been in culture for 30 to 40 days using the procedure described in Example 6. EGF-responsive cells began to proliferate after a few DIV and formed floating spheres that were passaged a second time after 30 to 40 DIV.

Thirty to 60 days after passage two or three, 2–3 ml aliquots containing media and pass 2 spheres were taken from the tissue culture flasks and plated onto 35 mm culture dish. Single spheres were placed onto poly-L-ornithine coated glass coverslips in DMEM/F-12/HM medium containing EGF. Spheres immediately attached to the substrate and within the first 24–48 hours cells begin to migrate from the sphere. At 14 DIV cells continued to proliferate and migrate resulting in an increase in the diameter of the transferred sphere. By 30 DIV, a large number of cells had been generated from the original sphere and had migrated at a similar rate from the center producing a concentric circle of associated cells. At the periphery, the majority of the cells were one cell layer thick while closer to the center there were denser regions of cells.

Forebrain regions from eight week old tissue produced no spheres, while spheres were observed from hindbrain tissue in two of the four eight week old samples. For the nine week old fetuses, spheres were generated from forebrain region in two of the four samples and in two of the three hindbrain regions which were received. The twelve week old fetus contained only hindbrain tissue and spheres were produced.

Spheres generated from primary culture or pass 1 spheres were removed from the tissue culture flask, without inducing differentiation, and plated onto poly-L-ornithine coated glass coverslips in DMEM/F-12/HM medium for two hours to allow the spheres to attach to the substrate. Coverslips were removed and processed for indirect immunohistochemistry. Immunostaining with antibodies directed against neurofilaments (168 kDa) or GFAP did not identify any immunoreactive (IR) cells. However, nearly all of the cells were immunoreactive with an antibody that recognizes human nestin.

Thirty to 45 days after being plated onto the poly-L-ornithine coated substrate, cells were fixed and processed for indirect immunocytochemical analysis with antibodies directed against: MAP-2, Tau-1, neurofilament 168 kDA, GABA, substance P (neuronal markers); GFAP (astrocyte marker); O4 and MBP (oligodendrocyte markers). Numerous MAP-2 and Tau-1-IR cell bodies and processes were identified in addition to a large number of Tau-1-IR fibers. While there was no indication of substance P immunoreactivity, GABA-IR cell bodies with long branched processes were seen. Neurofilament-IR cells were strongly IR for GFAP. O4-IR cells with an O-2A morphology and an oligodendrocyte morphology were present. MBP-IR (found on oligodendrocytes) was also seen throughout the cultures.

EXAMPLE 10: Proliferation of Adult Monkey (Rhesus) Neural Stem Cells and Differentiation of the Neural Stem Cell Progeny

The conus medularis was removed from an adult male monkey (Rhesus) and hand cut with scissors into 1-mm sections and transferred into artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (pH 7.35, approx. 280 mOsmol), aerated with 95% O_2–5% CO_2 at room temperature. After 15 min, the tissue sections were transferred to a spinner flask (Bellco Glass) with a magnetic stirrer filled with low-Ca^{2+} aCSF containing 124 mM NaCl, 5 mM KCl, 3.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (pH 7.35, approx. 280 mOsmol), aerated with 95% O_2–5% CO_2 at 32° to 35° C., containing 1.33 mg/ml of trypsin (9000 BAEE units/mg), 0.67 mg/ml of hyaluronidase (2000 units/mg) and 0.2 mg/ml of kynurenic acid. After 90 min, tissue sections were transferred to normal aCSF for 5 min prior to trituration. Tissue was transferred to DMEM/F-12 (1:1, Gibco) medium containing 0.7 mg/ml ovomucoid (Sigma) and triturated mechanically with a fire-narrowed pasteur pipet.

Cells were plated (1000 viable cells per plate) in non-coated 35 mm culture dishes (Costar) containing Complete Medium and 20 ng/ml EGF (human recombinant from Gibco/BRL). After 7 to 10 days in culture, floating spheres

were transferred with wide-bore pipets onto laminin (15 μg/ml)(Sigma)-coated glass coverslips in 24-well culture dishes. EGF@20 ng/ml was added to the medium. Spheres attached to the substrate and cells within the sphere continued to proliferate. After 14 to 21 days in vitro (DIV), the cells were probed by indirect immunocytochemistry for the presence of neuron, astrocytes and oligodendrocytes. All three cell types were identified.

EXAMPLE 11: Proliferation of Adult Human Neural Stem Cells and Differentiation of the Neural Stem Cell Progeny

During a routine biopsy, normal tissue was obtained from a 65 year old female patient. The biopsy site was the right frontal lobe, 6 mm from the tip of the frontal/anterior horn of the lateral ventricle. The tissue was dissociated using the procedure outlined in Example 2 and cultured in Complete Medium with EGF and FGF-2 (20 ng/ml of each growth hormone), in T25 flasks (Nunclon). The flasks were examined every 2–3 days for neurosphere formation. Clonally-derived cells were passaged using single sphere dissociation: single neurospheres were triturated 100× in sterile aliquot tubes containing 200 μl of the media/hormone/EGF-FGF-2 solution before culturing in 24- or 96-well plates. First-passage neurospheres were plated on poly-ornithine and laminin coated coverslips and allowed to plate down for 14 days in media/hormone/EGF+FGF-2. Some first passage neurospheres were plated on laminin (20 μg/ml) and poly-ornithine coated coverslips in media/hormone mix for 19 hours, then processed for nestin staining as outlined in Example 7. Nestin staining indicated that the neurospheres, prior to the induction of differentiation (as described below) were nestin positive, indicative of the presence of immature undifferentiated cells.

Pass one human neurospheres were plated on a laminin coated substrate (see above). After 14 days, the cultures received a media change to media/hormone mix plus 1% FBS and were allowed to differentiate for 7 days. Immunocytochemical analysis was then performed to determine different neural phenotypes. The differentiated cells were fixed with 4% paraformaldehyde in PBS for 20 minutes. The coverslips were washed three times (five minutes each) in PBS. For triple label immunocytochemistry, the cells were permeabilized for 5 minutes in 0.3% TRITON®-X in PBS followed by 2 washes with PBS. A first set of primary antibodies, MAP-2 (mouse monoclonal, 1:1000, Boerhinger Mannheim) and GFAP (Rabbit polyclonal, 1:300, BTI), used to determine the presence of neurons and astrocytes respectively, were mixed in 10% normal goat serum in PBS. The cells were incubated at 37° C. for 2 hours and then washed 3 times in PBS. A first set of secondary antibodies, goat anti-mouse rhodamine (Jackson Immuno Research) and goat anti-rabbit FITC (IgG, 1:100 Jackson Immuno Research) were mixed in PBS. The cells were incubated for 30 minutes at 37° C. and then rinsed three times with PBS. The second primary antibody, O4 (mouse monoclonal IgM, 1:100) for oligodendrocytes, was mixed in 10% normal goat serum in PBS. The cells were incubated for 2 hours at 37° C. The second set of secondary goat anti-mouse AMCA IgM (1:100 Jackson Immuno Research) was mixed in PBS and cells were incubated for 30 minutes at 37° C. The cells were then rinsed twice in PBS and then in double distilled water before mounting with Fluorosave.

EXAMPLE 12: Screening for the trkB Receptor on Neural Stem Cell Progeny

The expression of the trk family of neurotrophin receptors in EGF-generated neurospheres was examined by northern blot analysis. Total mRNA was isolated from mouse and rat striatal sGF-generated neurospheres. Both rat and mouse neurospheres expressed high levels of trkB receptor mRNA, but did not express trk nor trkc mRNA. In preliminary experiments, single EGF-generated mouse neurospheres were plated on poly-L-ornithine coated glass coversips and cultured in the absence or presence of 10ng/ml of BDNF. When examined after 14–28 days in vitro, neurospheres plated in the presence of BDNF contained NSE(+) cells with extensive and highly branched processes; well-developed NSE(+) cells were not observed in the absence of BDNF. Activation of the trkB receptor on EGF-generated neurospheres may enhance differentiation, survival of and/or neurite outgrowth from newly generated neurons.

EXAMPLE 13: Screening for the GAP-43 Membrane Phosphoprotein on Neural Stem Cell Progeny

Growth-associated protein (GAP-43) is a nervous system-specific membrane phosphoprotein which is down-regulated during development. Originally, GAP-43 was though to be neuron-specific, however, recent reports indicate that this protein may be at least transiently expressed during development in some astrocytes, oligodendrocytes and in Schwann cells. At present, the role of GAP-43 in macroglia is not known. The transient expression of GAP-43 in glial cells generated from the EGF-responsive stem cells derived from embryonic and adult murine striatum was investigated. Glial cell (astrocyte and oligodendrocyte) differentiation was induced by plating neural stem cell progeny in a medium containing 1% FBS with no EGF. The cells were then probed with specific antibodies for GAP-43,nestin, GFAP, O4, and GalC. In order to identify cells expressing GAP-43,the antibodies were pooled in various combinations using dual-label immunofluorescence methods.

During the first two days post plating, there was a low to moderate level of GAP43 expression in almost all cells (flat, bipolar and stellate), but by 3–4 days post-plating, the level of GAP-43 expression became restricted to the bipolar and stellate cells. At 4 days the majority of GAP-43-expressing cells co-labelled with the oligodendrocyte markers O4 and GalC although GFAP and GAP-43 was coexpressed in a number of cells. At one week post-plating however, essentially all of the GFAP-expressing astrocytes no longer expressed GAP-43 while the majority of the O4 and GalC-expressing cells continued to express GAP-43. At 7–10 days, these oligodendrocytes began to express MBP and lose the expression of GAP-43. The EGF-responsive stem cells may represent a useful model system for the study of the role of GAP-43 in glial and neuronal development.

EXAMPLE 14: Treatment of Neurodegenerative Disease Using Progeny of Human Neural Stem Cells Proliferated In Vitro

Cells are obtained from ventral mesencephalic tissue from a human fetus aged 8 weeks following routine suction abortion which is collected into a sterile collection apparatus. A 2×4×1 mm piece of tissue is dissected and dissociated as in Example 1. Neural stem cells are then proliferated as in Example 4. Neural stem cell progeny are used for neurotransplantation into a blood-group matched host with a neurodegenerative disease. Surgery is performed using a BRW computed tomographic (CT) stereotaxic guide. The patient is given local anesthesia suppiemencea with intravenously administered midazolam. The patient undergoes CT scanning to establish the coordinates of the region to

receive the transplant. The injection cannula consists of a 17-gauge stainless steel outer cannula with a 19-gauge inner stylet. This is inserted into the brain to the correct coordinates, then removed and replaced with a 19-gauge infusion cannula that has been preloaded with 30 μl of tissue suspension. The cells are slowly infused at a rate of 3 μl/min as the cannula is withdrawn. Multiple stereotactic needle passes are made throughout the area of interest, approximately 4 mm apart. The patient is examined by CT scan postoperatively for hemorrhage or edema. Neurological evaluations are performed at various post-operative intervals, as well as PET scans to determine metabolic activity of the implanted cells.

EXAMPLE 15: Remyelination of Myelin Deficient Rats Using Neural Stem Cell Progeny Proliferated In Vitro

Embryonic day 15 (E15) Sprague Dawley rats and E14–15 mice were obtained and the neural tissue was prepared using the methods described in Example 1. The cells were suspended in Complete Medium with 16–20 ng/ml EGF (purified from mouse submaxillary, Collaborative Research) or TGFα (human recombinant, Gibco). The cells were seeded in a T25 culture flask and housed in an incubator at 37° C., 100% humidity, 95% air/5% CO_2 and proliferated using the suspension culture method of Example 4. Cells proliferated within 3–4 days and, due to lack of substrate, lifted off the floor of the flask and continued to proliferate in suspension forming neurospheres.

After 6–8 days in vitro (DIV) the neurospheres were removed, centrifuged at 400 r.p.m. for 2–5 minutes, and the pellet mechanically dissociated into individual cells with a fire-polished glass pasteur pipet. Cells were replated in the growth medium where proliferation of the stem cells and formation of new neurospheres was reinitiated.

Litters of first day postnatal myelin deficient rats were anesthetized using ice to produce hypothermia. Myelin deficiency is an X-linked trait and thus only one half of the males in any litter are affected. Therefore, only the males were used for these studies. Once anesthetized, a small rostral to caudal incision was made at the level of the lumbar enlargement. The muscle and connective tissue was removed to expose the vertebral laminae. Using a fine rat tooth forceps, one lamina at the lumbar enlargement was removed and a small cut is made in the dura mater to expose the spinal cord.

A stereotaxic device holding a glass pipet was used to inject a 1 μl aliquot of the cell suspension (approximately 50,000 cells/μl) described above. The suspension is slowly injected into a single site (although more could be done) in the dorsal columns of the spinal cord. As controls, some of the animals were sham-injected with sterile saline. The animals were marked by clipping either toes or ears to distinguish between both experimental groups. Following injection of the cell suspension, the wound was closed using sutures or stainless steel wound clips and the animals were revived by warming on a surgical heating pad and then returned to their mother.

The animals were allowed to survive for two weeks post-injection and were then deeply anesthetized with nembutal (150 mg/kg) and perfused through the left ventricle. The spinal cords were removed and the tissue examined by light and electron microscopy. Patches of myelin were found in the dorsal columns of the recipients of both rat and mouse cells, indicating that neural stem cells isolated from rat and mouse neural tissue can differentiate into oligodendroglia and are capable of myelination in vivo.

Because the myelin deficient rat spinal cord is almost completely devoid of myelin, myelin formed at or near the site of injection is derived from the implanted cells. It is possible that the process of injection will allow for the entry of Schwann cells (myelinating cells of the PNS) into the spinal cord. These cells are capable of forming myelin within the CNS but can be easily distinguished from oligodendrocytes using either light microscopy or immunocytochemistry for CNS myelin elements. There is usually a very small amount of CNS myelin within the myelin deficient rat spinal cord. This myelin can be distinguished from normal donor myelin based on the mutation within the gene for the major CNS myelin protein, proteolipid protein (PLP). The myelin deficient rat myelin is not immunoreactive for PLP while the donor myelin is.

EXAMPLE 16: Remyelination in Human Neuromyelitis Optica

Neuromyelitis optica is a condition involving demyelination of principally the spinal cord and optic nerve. Onset is usually acute and in 50% of the cases death occurs within months. The severity of demyelination as well as lesion sites can be confirmed by magnetic resonance imaging (MRI).

Neural stem cell progeny are prepared from fetal human tissue by the methods of Example 9 or 14. Cells are stereotactically injected into the white matter of the spinal cord in the vicinity of plaques as visualized by MRI. Cells are also injected around the optic nerve as necessary. Booster injections may be performed as required.

EXAMPLE 17: Remyelination in Human Pelizaeus-Merzbacher Disease

Pelizaeus-Merzbacher disease is a condition involving demyelination of the CNS. The severity of demyelination as well as lesion sites can be confirmed by magnetic resonance imaging (MRI).

Neural stem cell progeny are prepared from fetal human tissue by the methods of Examples 9 or 14. Cells are stereotactically injected into the white matter of the spinal cord in the vicinity of plaques as visualized by MRI. Cells are also injected around the optic nerve as necessary. Booster injections may be performed as required.

EXAMPLE 18: Genetic Modification of Neural Stem Cell Progeny

Cells proliferated as in Examples 3 or 4 are transfected with expression vectors containing the genes for the FGF-2 receptor or the NGF receptor. Vector DNA containing the genes are diluted in 0.1X TE (1 mM Tris pH 8.0, 0.1 mM EDTA) to a concentration of 40 μg/ml. 22 μl of the DNA is added to 250 μl of 2X HBS (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4.2H_2O$, 12 mM dextrose, 50 mM HEPES) in a disposable, sterile 5 ml plastic tube. 31 μl of 2M $CaCl_2$ is added slowly and the mixture is incubated for 30 minutes at room temperature. During this 30 minute incubation, the cells are centrifuged at 800 g for 5 minutes at 4° C. The cells are resuspended in 20 volumes of ice-cold PBS and divided into aliquots of 1×10^7 cells, which are again centrifuged. Each aliquot of cells is resuspended in 1 ml of the DNA-$CaCl_2$ suspension, and incubated for 20 minutes at room temperature. The cells are then diluted in growth medium and incubated for 6–24 hours at 37° C. in 5%–7% CO^2. The cells are again centrifuged, washed in PBS and returned to 10 ml of growth medium for 48 hours.

The transfected neural stem cell progeny are transplanted into a human patient using the procedure described in

Example 14, or are used for drug screening procedures as described in the examples below.

EXAMPLE 19: Genetic Modification of Neural Stem Cell Progeny With a Retrovirus Containing the Bacterial B-Galactosidase Gene

Neural stem cell progeny were propagated as described in Example 4. A large pass-1 flask of neurospheres (4–5 days old) was shaken to dislodge the spheres from the flask. The flask was spun at 400 r.p.m. for 3–5 minutes. About half of the media was removed without disturbing the neurospheres. The spheres and the remaining media were removed, placed into a Falcon 12 ml centrifuge tube, and spun at 600 r.p.m. for 3–5 minutes. The remaining medium was removed, leaving a few hundred microliters.

A retrovirus which contained the bacterial B-galactosidase gene was packaged and secreted, in a replication-deficient fashion, by the CRE BAG2 cell line produced by C. Cepko. A day after the CRE cells reached confluence, the cells were washed with PBS and the retrovirus was collected in DMEM/F12/HM/20 ng/ml EGF for four days. The virus-containing media was filtered through a 0.45 μm syringe filter. The neurospheres were resuspended in the virus-containing media, transferred to a large flask, and left in an incubator overnight at 37° C. The next day, the contents of the flask were transferred to a 12 ml centrifuge tube and spun at 800 r.p.m. The cells were resuspended in EGF-containing media/HM, dissociated into single cells, and counted. The cells were replated in a large flask at 50,000 cells/ml in a total of 20 mls.

Four days later, transformed cells were selected with G418 at a concentration of 300 μg/ml. Transformed spheres were plated on a poly-ornithine coated glass coverslip in a 24-well plate. After the neurospheres adhered to the plate, the cells were fixed with 0.1% glutaraldehyde for 5 minutes at 4° C. After the cells were fixed, they were washed twice-with PBS for 10 minutes. The cells were then washed with 0.1% TRITON® in PBS for 10–15 minutes at room temperature. A 1 mg/ml X-Gal solution was added to each well and incubated overnight at 37° C. After incubation overnight, the cells were washed three times with PBS for 10 minutes each and observed for any reaction products. A positive reaction resulted in a blue color, indicating cells containing the transferred gene.

EXAMPLE 20: Proliferation of Neural Stem Cells from Transgenic Mice

Transgenic mice were produced using standard pronuclear injection of the MBP-lacZ chimeric gene, in which the promoter for MBP directs the expression of *E. coli* B-galactosidase (lacZ). Transgenic animals were identified by PCR using oligonucleotides specific for lacZ.

Neurospheres were prepared from E15 transgenic mice and DNA negative littermates using the procedures set forth in Example 4. The neurospheres were propagated in the defined culture medium in the presence of 20 ng/ml EGF and were passaged weekly for 35 weeks. For passaging, the neurospheres were harvested, gently centrifuged at 800 RPM, and mechanically dissociated by trituration with a fire-polished Pasteur pipet. At various passages, the cells were induced to differentiate into oligodendrocytes, astrocytes, and neurons by altering the culture conditions. The free-floating stem cell clusters were gently centrifuged, resuspended in the same base defined medium without EGF and with 1% FBS and plated on poly ornithine-treated glass coverslips to promote cell attachment. The clusters attach firmly to the glass, and the cells slow or stop dividing and begin to differentiate.

The identification of various cell types was accomplished using immunofluorescence microscopy with antibodies specific for neurons (MAP-2, NF-L, and NF-M), astrocytes (GFAP) and oligodendrocytes and oligodendrocyte precursors (A2B5, O_1, O_4, Gal C, and MBP). One to 14 days post-plating, the cells on the coverslips were incubated unfixed, for 30 minutes at room temperature with the primary antibodies O1, O4, GalC, and A2B5 (supernatants) diluted in minimal essential medium with 5% normal goat serum and 25 mM HEPES buffer, pH 7.3 (MEM-HEPES, NGS). Following the primary antibodies, the coverslips were gently washed 5 times in rhodamine-conjugated secondary antibodies (Sigma) diluted in MEM-HEPES, NGS. The coverslips were then washed 5 times in MEM-HEPES and fixed with acid alcohol (5% glacial acetic acid/95% ethanol) at 20° C. The coverslips were then washed 5 times with MEM-HEPES, and either mounted and examined using fluorescence microscopy or immmunoreacted with rabbit polyclonal antisera raised against GFAP, nestin, MBP, or proteolipid protein (PLP). When subjected to a second round of immunolabeling, the coverslips were incubated first for 1 hour with 5% normal goat serum (NGS) in 0.1M phosphate buffer with 0.9% NaCl at pH 7.4 (PBS) and then incubated in rabbit primary antibodies diluted in NGS for 1–2 hours at room temperature. The coverslips were washed 3 times with PBS and then incubated with the appropriate secondary antibody conjugates diluted in NGS, washed again with PBS and then finally mounted on glass microscope slides with Citifluor antifadent mounting medium and examined using a fluorescence microscope. In cases were they were not incubated first with the monoclonal antibody supernatants, the coverslips were fixed for 20 minutes with 4% paraformaldehyde in PBS (pH 7.4), washed with PBS, permeabilized with 100% ethanol, washed again with PBS and incubated with 5% NGS in PBS for 1 hour. The primary antibodies and secondary antibody conjugates were applied as outlined above.

The neural stem cells derived from the transgenic animals were indistinguishable from non transgenic stem cells in their potential for differentiation into neurons, astrocytes, and oligodendrocytes. The MBP promoter directed the expression of the B-galactosidase reporter gene in a cell-specific and developmentally appropriate fashion. The transgene expression is highly stable as oligodendrocytes derived from late passage MBP-lacZ neurospheres (20 passages), expressed the B-galactosidase gene. Thus, transgenically marked neurospheres are likely to be an excellent source of cells for glial cell transplantation.

EXAMPLE 21: Genetic Modification of Neural Stem Cell Progeny Using Calcium Phosphate Transfection

Neural stem cell progeny are propagated as described in Example 4. The cells are then infected using a calcium phosphate transfection technique. For standard calcium phosphate transfection, the cells are mechanically dissociated into a single cell suspension and plated on tissue culture-treated dishes at 50% confluence (50,000–75,000 cells/cm^2) and allowed to attach overnight.

The modified calcium phosphate transfection procedure is performed as follows: DNA (15–25 μg) in sterile TE buffer (10 mM Tris, 0.25 mM EDTA, pH 7.5) diluted to 440 μl with TE, and 60 μl of 2M $CaCl_2$ (pH to 5.8 with 1M HEPES buffer) is added to the DNA/TE buffer. A total of 500 μl of

2×HeBS (HEPES-Buffered saline; 275 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4,, 12 mM dextrose, 40 mM HEPES buffer powder, pH 6.92) is added dropwise to this mix. The mixture is allowed to stand at room temperature for 20 minutes. The cells are washed briefly with 1×HeBS and 1 ml of the calcium phosphate precipitated DNA solution is added to each plate, and the cells are incubated at 37° for 20 minutes. Following this incubation, 10 mls of complete medium is added to the cells, and the plates are placed in an incubator (37° C., 9.5% CO_2) for an additional 3–6 hours. The DNA and the medium are removed by aspiration at the end of the incubation period, and the cells are washed 3 times with complete growth medium and then returned to the incubator.

EXAMPLE 22: Genetically Modified Neural Stem Cell Progeny Expressing NGF

Using either the recombinant retrovirus or direct DNA transfection technique, a chimeric gene composed of the human CMV promoter directing the expression of the rat NGF gene is introduced into the neurosphere cells. In addition, the vector includes the *E. coli* neomycin resistance gene driven off of a viral promoter. After 2 weeks of G418 selection, the cells are cloned using limiting dilution in 96-multi-well plates and the resulting clones are assayed for neurotrophin protein expression using a neurotrophin receptor (trk family) autophosphorylation bioassay.

Clones expressing high levels of NGF are expanded in T-flasks prior to differentiation. The cells are then removed from the EGF-containing complete medium and treated with a combination of serum and a cocktail of growth factors to induce astrocyte differentiation. The astrocytes are again assayed for NGF expression to ensure that the differentiated cells continue to express the trophic factors. Astrocytes that secrete NGF are then injected into fimbria/fornix lesioned rat brains immediately post-lesioning in order to protect the cholinergic neurons. Control astrocytes that do not secrete NGF are injected into similarly lesioned animals. The sparing of cholinergic neurons in the lesion model is assessed using immunocytochemistry for ChAT, the marker for these cholinergic neurons.

EXAMPLE 23: Genetically Modified Neural Stem Cell Progeny Expressing CGAT

Recently, a novel chromaffin granule amine transporter (CGAT) cDNA has been described by Liu et al. (*Cell.* 70:539–551 (1992)), which affords resistance to the neurotoxin MPP+ in Chinese hamster ovary (CHO) cells in vitro. Because dopaminergic neurons from the substantia nigra are specifically killed by MPP+, CGAT gene expression in genetically modified neural stem cell progeny may improve viability of the cells after they are implanted into the Parkinsonian brain. Neural stem cell progeny are propagated as in Example 4. The cells are mechanically dissociated and plated on plastic dishes and infected with a retrovirus containing the CGAT cDNA. The expression of the CGAT cDNA (Liu et al. supra) is directed by a constitutive promoter (CMV or SV40, or a retroviral LTR) or a cell-specific promoter (TH or other dopaminergic or catecholaminergic cell-specific regulatory element or the like). The cells are screened for the expression of the CGAT protein. Selected cells can then be differentiated in vitro using a growth factor or a combination of growth factors to produce dopaminergic or predopaminergic neurons.

EXAMPLE 24: 3H-Thymidine Kill Studies Identify Presence of Constitutively Proliferating Population of Neural Cells in Subependymal Region

Adult male CD1 mice received a series of intraperitoneal injections of 3H-thymidine (0.8 ml per injection, specific activity 45–55 mCi/mmole, ICN Biomedical) on day 0 (3 injections, 1 every 4 hours) in order to kill the constitutively proliferating subependymal cells. On day 0.5, 1, 2, 4, 6, 8 or 12, animals received 2 BrdU injections 1 hour apart (see Example 25) and were sacrificed 0.5 hour after the last injection.

It was observed that 10% of the cells were proliferating on day 1 post-kill, and by 8 days the number of proliferating cells had reached 85%, which was not statistically significantly different from control values. Animals were sacrificed and the brains were removed and processed as described in Example 10.

In a second group of animals, 3H-thymidine injections were given on day 0 (3 injections, 1 every 4 hours), followed by an identical series of injections on day 2 or 4. Animals were allowed to survive for 8 days following the second series of injections (days 9, 10 and 12 respectively) at which time they received 2 injections of BrdU and were sacrificed 0.5 hours later. Animals were sacrificed and the brains were removed and processed as described in Example 25.

After the second series of injections on day 2 only 45% of the proliferating population had returned relative to control values. This indicates that the second series of injections given on day 2 had killed the stem cells as they were recruited to the proliferating mode. The second series of injections given on day 4 resulted in a return to control values by day 8 suggesting that by this time, the stem cells were no longer proliferating and hence were not killed by the day 4 series of injections.

EXAMPLE 25: BrdU Labeling Studies Identifv Presence of Constitutively Proliferating Population of Neural Cells in Subependymal Region

Adult male CD1 mice (25–30 g, Charles River) were injected intraperitoneally (i.p.) with bromodeoxyuridine (BrdU, Sigma, 65 mg/kg) every 2 hours for a total of 5 injections in order to label all of the constitutively proliferating cells in the subependyma lining the lateral ventricles in the forebrain. One month later, animals were sacrificed with an overdose of sodium pentobarbital and transcardially perfused using 4% paraformaldehyde. The brains were removed and post-fixed overnight in 4% paraformaldehyde with 20% sucrose. Brain sections were cut on a cryostat (30 um) and collected in a washing buffer [0.1M phosphate buffered saline (PBS) pH 7.2 with 1% normal horse serum and 0.3% TRITON® X100]. Sections were incubated in 1M HCl at 60° C. for 0.5 hours then washed 3 times (10 minutes each) in washing buffer. Following the final wash, sections were incubated in anti-BrdU (Becton Dickinson, 1:25) for 45 hours at 4° C.. After incubation in the primary antibody, sections were washed 3 times and incubated for 1 hours in biotinylated horse-anti-mouse secondary antibody Dimension Lab, 1:50) at room temperature followed by another 3 washes. The sections were then incubated for 1 hour in avidin conjugated FITC (Dimension Lab, 1:50) at room temperature and washed a final 3 times. Sections were mounted on gelatin coated slides, air-dried and coversnipped with Fluoromount. Slides were examined for BrdU positive cells using a NIKON fluorescent microscope. The number of BrdU positive cells was counted with in the subependyma surrounding the lateral ventricles in 8 samples in sections between the closing of the corpus callosum rostrally and the crossing of the anterior commissure caudally. It was found that 31 days following the series of BrdU injections, 3% of the subependymal cells were still labeled compared to control animals sacrificed immediately following the series of injections (control 100%).

EXAMPLE 26: 3H-Thymidine Kill Studies Identify Presence of Relively Quiescent Neural Stem Cells in Subependymal Region

Adult male CD1 mice were divided into 4 groups. Group A animals received a series of 3H-thymidine injections on day 0 (3 injections, 1 every 4 hours). Animals in groups B and C received a series of 3H-thymidine injections on day 0 followed by a second series of injections on day 2 or 4. Group D animals received injections of physiological saline instead of 3H-thymidine over the same time course a s group A. Animals from all groups were sacrificed by cervical dislocation 16–20 hours following the last series of injections. Brains were removed and neural tissue obtained from the subependyma surrounding the lateral ventricles in the forebrain was dissociated and the neural cells cultured as described in Example 5. At 6 and 8 days in vitro, the total number of spheres was counted in each of the 35 mm wells.

Control animals that received a series of saline injections formed the same number of spheres as animals that received 3H-thymidine on day 0 (which kills the normally proliferating subependymal cells). This indicates that the constitutively proliferating subependymal cells are not the source of stem cells isolated in vitro. Animals that received a second series of injections on day 2 formed 45% the number of spheres (similar to the number of proliferating subependymal cells observed in vivo). When a second series of injections was done on day 4, the number of spheres formed in vitro was not significantly different from control values, again correlating with the in vivo findings. Taken together, this data indicates that the multipotent spheres, which are isolated in vitro in the presence of EGF, are formed from the relatively quiescent stem cell population within the subependyma in vivo.

EXAMPLE 27: In Vivo Proliferation of Neural Stem Cells of Lateral Ventricle

A replication incompetent retrovirus containing the β-galactosidase gene [as described in Walsh and Cepko, *Science* 241:1342, (1988)] was injected into the forebrain lateral ventricles of CD1 adult male mice (25–30 g from Charles River). The injected retrovirus was harvested from the BAG cell line (ATCC CRL-9560) according to the method of Walsh and Cepko (supra). Mice were anesthetized using 65 mg/kg, i.p. sodium pentobarbital. Unilateral stereotactic injections of 0.2–1.0 μl of retrovirus were injected into the lateral ventricle using a 1 μl Hamilton syringe. The coordinates for injection were AP+4.2 mm anterior to lambda, L±0.7 mm, and DV–2.3 mm below dura, with the mouth bar at –2 mm below the interaural line.

On the same day as, one day, or six days following the retrovirus injection, an infusion cannulae attached to a 0.5 μl/hour ALZET osmotic mini-pumps filled with 3.3–330 μg/ml of EGF were surgically implanted into the lateral ventricles at the identical stereotactic coordinates as stated above. The infusion cannula kits were obtained from ALZA. The infusion cannulae were cut to 2.7 mm below the pedestal. The pumps were secured to the mouse skull by use of acrylic cement and a skull screw contralateral and caudal to the injection site. The osmotic mini-pump was situated subcutaneously under and behind the armpit of the left front paw and connected to the infusion cannula by the means of polyethylene tubing.

Six days following initiation of EGF infusion the animals were sacrificed with an overdose of sodium pentobarbital. Mice were transcardially perfused with 2% buffered paraformaldehyde, and the brains were excised and post fixed overnight with 20% sucrose in 2% buffered paraformaldehyde. Coronal slices were prepared with –20 celsius cryostat sectioning at 30 μm. Slices were developed for β-gal histochemistry as per Morshead and Van der Kooy (supra).

Under these conditions, regardless of the day post retrovirus injection, infusion of EGF resulted in an expansion of the population of β-gal labelled cells from an average of 20 cells per brain up to an average of 150 cells per brain and the migration of these cells away from the lining of the lateral ventricles. Infusion of FGF-2 at 33 μg/ml resulted in an increase in the number of β-gal labelled cells, but this increase was not accompanied by any additional migration. Infusion of EGF and FGF together resulted in an even greater expansion of the population of β-gal labelled cells from 20 cells per brain to an average of 350 cells per brain.

These results indicate that FGF may be a survival factor for relatively quiescent stem cells in the subependyma layer, whereas EGF may act as a survival factor for the normally dying progeny of the constitutively proliferating population. The synergistic increase in β-galactosidase cell number when EGF and FGF are infused together further reflects the direct association between the relatively quiescent stem cell and the constitutively proliferating progenitor cell.

EXAMPLE 28: In Vivo Proliferation of Neural Stem Cells of the Third and Fourth Ventricles and the Central Canal

A retroviral construct containing the β-galactosidase gene is microinjected (as in Example 27) into the III ventricle of the diencephalon, IV ventricle of the brain stem and central canal of the spinal cord. Minipumps containing EGF and FGP are then used to continuously administer growth factors for six days (as in Example 27) into the same portion of the ventricular system that the retroviral construct was administered. This produces an increase in the number of β-galactosidase producing cells which survive and migrate out into the tissue near the III ventricle, IV ventricle and central canal of the spinal cord forming new neurons and glia.

EXAMPLE 29: In Vivo Modification and Proliferation of Neural Stem Cells and Differentiation of Neural Stem Cell Progeny of the Lateral Ventricle

A retroviral construct containing the TH gene as well as the β-galactosidase gene is microinjected into the adult lateral ventricle as in Example 27. Mini-pumps containing EGF, FGF, or EGF and FGF together are then used to continuously administer the growth factor(s) into the lateral ventricle for 6 days as in Example 27. As the infected subependymal cells migrate out into the striatum they differentiate into neuronal cells that produce dopamine as measured directly by immunofluorescence with an antibody and (from a direct functional assay) by the ability to overcome the rotational bias produced by unilateral 6-hydroxydopamine lesions.

EXAMPLE 30: In Vivo Infusion of Growth Factors into Ventricles to Obtain Elevated Numbers of Neural Stem Cells

Adult male CD_1 albino mice (30–35 g) from Charles River were anaesthetized with sodium pentobarbital (0.40 mL of a 10% solution) and placed in a stereotaxic apparatus. The dorsal aspect of the skull was exposed with a longitudinal incision. Cannulas were inserted into the fourth ven-

tricle (stereotaxic coordinates A/P −7.0, L±0.3 D/V−5.8), cerebral aqueduct (A/P −4.8 L±D/V−2.6), or central canal (D/V−1.5). The cannulae were attached with sterile tubing to subcutaneous positioned ALZET osmotic mini-pumps containing 25 μg/mL EGF (Becton 40001) and/or 25 μg/mL FGF-2 (R&D Systems 233-FB). Pumps containing sterile saline plus 0.1% mouse albumin (Sigma A3134) were used as controls. The incisions were closed with dental cement. Six days following surgery mice were injected with 0.15 mL BrdU (Sigma B5002); 18 mg/mL in 0.007% NaOH/0.1M PBS) every 2 hours for 8 hours. They were killed 0.5 hours after the last injection with an anaesthetic overdose, and transcardially perfused with 10 mL of ice-cold sterile saline followed by 10 mL of ice-cold Bouin's fixative (5% glacial acetic acid, 9% formaldehyde, 70% picric acid). The cervical spinal cord region was dissected out and post-fixed overnight at 4° C. in Bouin's post-fixative solution (9% formaldehyde, 70% picric acid). The following day the tissue was cryoprotected by immersion in 10% sucrose for 2 hours, 20% sucrose for 2 hours, and 30% sucrose overnight. The tissue was frozen in powdered dry ice, mounted in Tissue-Tek (Miles 4583) at −18° C., and 30 μm serial sagittal sections were mounted onto gel-subbed glass slides. Each slide also contained one or more 30 μm coronal sections through the lateral ventricles from the brain of the same animal to serve as a positive control. Slides were kept at −80° C. until processed. Immunohistochemistry: Slides were rinsed in PBS 3×15 minutes in 0.1M PBS at room temperature, hydrolyzed with 1N HCl for 60 minutes at 37° C., rinsed for 3×15 minutes in 0.1M PBS at room temperature, placed in 6% H_2O_2 in methanol for 30 minutes at room temperature, rinsed for 3×15 minutes in 0.1M PBS at room temperature, and incubated in 10% normal horse serum (Sigma H-0146) in 0.1M PBS or 20 minutes at room temperature. Slides were incubated overnight at room temperature in anti-BrdU monoclonal antibody (Becton 7580) that was diluted 1:50 in 0.1M PBS containing 1.5% normal horse serum and 0.3% TRITON®. The following day the slides were rinsed in PBS for 3×10 minutes in 0.1M PBS at room temperature, incubated with biotinylated horse anti-mouse IgG (Vector BA-2000) for 2 hours at room temperature, rinsed for 3×15 minutes in 0.1M PBS at room temperature, incubated in ABC reagent (Vector PK-6100) for 2 hours at room temperature, rinsed for 3×15 minutes in 0.1M PBS at room temperature, and developed with DAB reagent for 2 to 4 minutes. The slides were coverslipped with Aqua Polymount (Polysciences 18606). The number of BrdU positive cells was counted per cervical spinal cord section. Some BrdU labelled cells were found in the saline control sections. Treatment with either EGF or FGF-2 resulted in a significant increase in the number of BrdU labelled cells seen compared to control. The combination of EGF plus FGF-2 produced even a greater amount of BrdU positive cells per section.

EXAMPLE 31: In Vivo Infusion of Growth Factors into Ventricles to Increase Yield of Neural Stem Cells That Proliferate In Vitro

EGF pumps were implanted as described in Example 27. Animals were sacrificed by cervical dislocation 6 days after the pump was implanted. Brains were removed and the stem cells isolated and counted as described in Example 5.

Animals infused with EGF into the lateral ventricles for 6 days prior to sacrifice and brain culturing had 4 times as many spheres forming after 9 days in vitro compared to control animals which received saline pumps for the same 6 day period. Thus, infusing EGF into the lateral ventricles in vivo prior to removal and dissociation of neural tissue, greatly increases the yield of stem cells which proliferate and form neurospheres in vitro.

EGF and FGF can be infused into the ventricles to further increase the yield of neural stem cells obtainable from the neural tissue. Neurospheres generated by this method are used as a source of donor cells for later transplantation into degenerated areas of human adult CNS. Neurospheres can also be proliferated accordingly from a patient's own CNS stem cells and transplanted back into the patient.

EXAMPLE 32: In Vivo Modification of Neural Cells with bcl-2 Gene

A retroviral construct containing the human bcl-2 gene and the β-galactosidase gene is microinjected into the adult mouse lateral ventricle. A control mouse is injected with a retroviral construct containing only the β-galactosidase gene. One of the two progeny of each of the constitutively proliferating subependymal cells of the adult lateral ventricle normally dies within a few hours after division. The bcl-2 gene product prevents the programmed death of cells in several other tissues. In the adult subependyma, single cells infected with both the β-galactosidase and bcl-2 genes are marked by expression of both these gene products. These cells are identified in brain tissue slices with antibodies specific to β-galactosidase and human Bcl-2. Proliferating infected subependymal cells so infected produce larger numbers of cells per clone relative to the control. Thus, Bcl-2 induces the survival of the one normally dying progeny of each division of a constitutively proliferating adult subependymal cell. Moreover, the bcl-2 infected progeny migrate out into striatal and septal tissue to produce new neurons and glia. This indicates that EGF and Bcl-2 act as a survival factors for the normally dying progeny of constitutively proliferating adult subependymal cells which generate new neurons and glia in vivo.

EXAMPLE 33: In Vivo Modification of Neural Cells with NGF Gene

A retroviral construct containing the NGF gene is microinjected using the procedure described in Example 27 to infect the constitutively proliferating adult subependymal cells of the lateral ventricle. Thus, these cells are used to produce an endogenous growth factor in the adult brain. Levels of NGF produced by the transfected cells are measured directly by radioimmunoassay and (from a direct functional assay) by rescue of basal forebrain cholinergic neurons in vivo after axotomy injury in the model developed by Gage and collaborators (P.N.A.S. 83:9231, 1986).

EXAMPLE 34: Generation of Dopamine Cells in the Striatum by the Administration of a Composition Comprising Growth Factors to the Lateral Ventricle

Adult male CD, mice were anesthetized and placed in a stereotaxic apparatus. A cannula, attached to an ALZET minipump, was implanted into a lateral ventricle of each animal. The minipumps were subcutaneously implanted and were used to deliver (a) conditioned medium (from the rat B49 glial cell line, obtained from D Schubert, Salk Institute) plus bFGF (R&D Systems, 25 μg/ml) plus heparan sulfate (Sigma, 10 IU/ml) (CMF) or (b) EGF (Chiron, 25 μg/ml) plus bFGF (25 μg/ml) plus heparan sulfate (10 IU/ml) plus 25% FBS (E+F+FBS) or (c) sterile saline solution (SAL) as a control, into the lateral ventricles. Once batch of animals was sacrificed one day after completion of the delivery

regimen and the others were sacrificed twenty days later. The subventricular zones (SVZs) of these mice were dissected out, separating the cannulated, and therefore treated, side from the noncannulated control sides. The substantia nigra (SN) region of these mice were also recovered. Total RNA was extracted from these tissues using the guanidium thiocyanate acid phenol method [Chomzynski and Sacchi, *Annal. Biochem.* 162: 156–159, (1987)]. The RNA was then reverse transcribed to produce cDNA. These cDNAs were subject to PCR using primers designed to bracket a 254 nucleotide region of the TH messenger RNA (mRNA) and thermal cycling conditions favoring quantitative amplification. The PCR products were electrophoresed on a 2% agarose gel and then capillary blotted onto a positively charged nylon membrane. Radioactively labelled cDNA probe to TH was hybridized to the filter and detected by autoradiography. The autoradiograph was scanned and analyzed by densitometry to obtain relative levels of mRNA for TH in the SVZs of the cannulated sides in response to the treatments in the non-cannulated control SVZs and in the SN. In animals analyzed one day after treatment, the administration of E+F+FBS produced an eleven-fold increase in the level of TH mRNA in the SVZ compared to that observed in response to CMF, which in turn was more than twice the level seen with SAL. Twenty one days after treatment, the amount of TH mRNA detected in response to treatment with E+F+FBS was approximately the same as that detected after one day, while CMF and SAL treated SVZs had TH mRNA levels which were below detectable limits and were indistinguishable from the non-cannulated SVZ controls. Under all treatments, the SN had measurable amounts of TH mRNA.

EXAMPLE 35: Detection of Dopaminerzic Cells in Striatal TYssue Using Dual Labelng

Male CD, mice (Charles River, approximately 4 to 6 weeks old) were given intraperitoneal injections of BrdU (Sigma, 120 mg/kg) at 2 hour intervals over a 24 hour period, in order to label mitotically active cells. A cannula attached to an ALZET minipump was then implanted unilaterally into a lateral ventricle of each animal in order to deliver compositions a–c (CMF, E+F+FBS, or sterile saline) described in Example 34.

Animals were sacrificed 24 hours after the administration of growth factors using a lethal dose of pentobarbital anesthetic. The animals were then perfused through the heart with 10 ml of ice could 4% paraformaldehyde solution. The brains were removed and tissue in the region extending from the olfactory bulb to the third ventricle, including the striatum, was dissected out and stored overnight at 4° C. in a 30% sucrose/4% paraformaldehyde solution. The tissue was then frozen on dry ice and kept at −70° C. until processed. 30 μm coronal sections were cut using a cryostat and the sections were placed in 12 well porcelain dishes, to which 400 μl of PBS had been added. Sections were rinsed with fresh PBS and incubated overnight with the following primary antibodies: anti-TH (rabbit polyclonal, 1:1000, Eugene Tech International Inc.; or 1:100, Pel-freeze) and mouse anti-BrdU (1:55, Amersham), prepared in PBS/10% normal goat serum/0.3 TRITON®X-100. Following three rinses in PBS, goat anti-rabbit rhodamine and goat anti-mouse fluorescein (Jackson) were applied in PBS for 50 minutes at room temperature. Sections were then washed three times (10 minutes each) in PBS, placed on glass slides, dried and then coverslipped using Fluorsave (Calbiochem #345789).

The location of dopaminergic neurons was determined by mapping the location of TH-immunoreactive (TH+) cells, or TH+ and BrdU+ cells in relation to the ventricles. In response to saline injections made into the lateral ventricles, the normal population of TH+ fibers were detected in the striatum but no TH+ cell bodies were detected in this region. CMF treatment resulted in the detection of TH+ cell bodies, in addition to the normal population of TH+ fibers, in the striatum and in the region of the third ventricle. E+F+FBS treatment had the most profound effect resulting in the detection of the most TH+ cell bodies. Several of the TH+ cell bodies were also BrdU positive.

EXAMPLE 36: Rat Model of Parkinson's Disease Measures the Effects of In Vivo Administration of Growth Factors

The 6-OHDA lesion rat model of Parkinson's disease is used to measure the effects of administering various combinations of growth factors to the lateral ventricle. Unilateral 6-OHDA lesions are performed in the rat model and rotation behavior is observed. Minipumps are subcutaneously implanted into the animals as described in Example 34. EGF (Chiron, 25 μg/ml) plus bFGF (25 μg/ml) plus heparan sulfate (10 IU/ml) plus 25% FBS is continuously administered to the lateral ventricle. Saline is administered to control animals. The ability to overcome the rotational bias produced by the unilateral 6-OHDA lesions is observed.

EXAMPLE 37: Screening of Drugs or Other Biological Agents for Effects on Multipotent Neural Stem Cells and Neural Stem Cell Progeny

A. Effects of BDNF on Neuronal and Glial Cell Differentiation and Survival

Precursor cells were propagated as described in Example 4 and differentiated using Paradigm 3 described in Example 7. At the time of plating the EGF-generated cells, BDNF was added at a concentration of 10 ng/mL At 3, 7, 14, and 21 days in vitro (DIV), cells were processed for indirect immunocytochemistry. BrdU labeling was used to monitor proliferation of the precursor cells. The effects of BDNF on neurons, oligodendrocytes and astrocytes were assayed by probing the cultures with antibodies that recognize antigens found on neurons (MAP-2, NSE, NF), oligodendrocytes (O4, GalC, MBP) or astrocytes (GFAP). Cell survival was determined by counting the number of immunoreactive cells at each time point and morphological observations were made. BDNF significantly increased the differentiation and survival of neurons over the number observed under control conditions. Astrocyte and oligodendrocyte numbers were not significantly altered from control values.

B. Effects of BDNF on the Differentiation of Neural Phenotypes

Cells treated with BDNF according to the methods described in Part A were probed with antibodies that recognize neural transmitters or enzymes involved in the synthesis of neural transmitters. These included TH, ChAT, substance P, GABA, somatostatin, and glutamate. In both control and BDNF-treated culture conditions, neurons tested positive for the presence of substance P and GABA. As well as an increase in numbers, neurons grown in BDNF showed a dramatic increase in neurite extension and branching when compared with control examples.

C. Identification of Growth-Factor Responsive Cells

Cells that are responsive to growth factor treatment were identified by differentiating the EGF-generated progeny as

described in Example 7, paradigm 3 and at 1 DIV adding approximately 100 ng/ml of BDNF. At 1, 3, 6, 12 and 24 hours after the addition of BDNF the cells were fixed and processed for dual label immunocytochemistry. Antibodies that recognize neurons (MAP-2, NSE, NF), oligodendrocytes (O4, GalC, MBP) or astrocytes (GFAP) were used in combination with an antibody that recognizes c-fos and/or other immediate early genes. Exposure to BDNF results in a selective increase in the expression of c-fos in neuronal cells.

D. Effects of BDNF on the Expression of Markers and Regulatory Factors During Proliferation and Differentiation

Cells treated with BDNF according to the methods described in Part A are processed for analysis of the expression of FGF-R1, as described in Example 39 or other markers and regulatory factors, as described in Example 40.

E. Effects of BDNF administration During Differentiation on the Electrophysiological Properties of Neurons

Neurons treated with BDNF during differentiation, according to the methods described in Part A, are processed for the determination of their electrophysiological properties, as described in Example 41.

F. Effects of Chlorpromazine on the Proliferation, Differentiation, and Survival of Growth Factor Generated Stem Cell Progeny

Chlorpromazine, a drug widely used in the treatment of psychiatric illness, is used in concentrations ranging from 10 ng/ml to 1000 ng/ml in place of BDNF in Examples 7A to 7E above. The effects of the drug at various concentrations on stem cell proliferation and on stem cell progeny differentiation and survival is monitored. Alterations in gene expression and electrophysiological properties of differentiated neurons are determined.

EXAMPLE 38: Stem Cell Proliferation Assay

Primary cells were obtained from E14 mice and prepared as detailed in Examples 1 and 4. Either EGF, EGF and FGF or EGF and BMP-2 were added to complete medium at a concentration of 20 ng/ml of each growth factor, with the exception of BMP-2 which was added at a concentration of 10 ng/ml. Cells were diluted with one of the prepared growth factor-containing media to a concentration of 25,000 cells/ml. 200 µl of the cell/medium combination were pipetted into each well of a 96-well place (Nuclon) with no substrate pretreatment. Cells were incubated under the same conditions as outlined in Example 4.

After 8–10 DIV the number of neurospheres was counted and the results tabulated. As cells grown in a combination of EGF and FGF produced significantly more neurospheres than cells grown in the presence of EGF alone. The combination of EGF and BMP-2 inhibited neurosphere development.

EXAMPLE 39: Comparison of Receptor and Growth Factor Expression in Undifferentiated vs. Differentiated Stem Cell-Derived Progeny by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Neurospheres were generated as described in Example 4, and some were differentiated as per Paradigm 1, Example 7. RNA from either undifferentiated or differentiated neurospheres was isolated according to the guanidinium thiocyanate acid phenol procedure of Chomzynski and Sacchi *Anal. Biochem.* 162: 156–159 1987)]. Complementary DNA (cDNA) was synthesized from total RNA using reverse transcriptase primed with oligo dT. Gene-specific primers were designed and synthesized and these primers were used in PCR to amplify cDNAs for different growth factors and growth factor receptors. Amplified material was run on agarose gels alongside molecular weight markers to ensure that PCR products were of the expected size, while the identity of PCR fragments was confirmed by restriction enzyme analysis and by sequencing [Arcellana-Panlilio, *Methods Enzymol.* 225: 303–328 (1993)]. An ethidium-stained agarose gel visualized via UV transillumination showed the detection of three growth factor receptor transcripts, namely EGF-R, FGF-R, and LIF-R, in undifferentiated and differentiated stem cell-derived progeny. Table I lists the primer sets analyzed and the results of undifferentiated and differentiated cells.

TABLE I

Primer Sets Analyzed

	Undifferentiated Cells	Differentiated Cells
Actin	+	+
NGF	+	nd
EGFr^m	+	+
bFGFr	+	+
LIFr^m	+	+
tyrosine hydroxylase	+	+
choline acetyltransferase^m	nd	+
cholecystokinin^m	nd	–
enkephalin^m	nd	+
tyrosine kinase-rA	+	+
tyrosine kinase-rB	+	+++++
tyrosine kinase-rC	+	+

r = receptor
m = derived from mouse
nd = no data available

EXAMPLE 40: Isolation of Novel Markers and Regulatory Factors Involved in Neural Stem Cell Proliferation and Differentiation

Neurospheres are generated as described in Example 4 using CNS tissue from CD, albino mice (Charles River). Some of these neurospheres are allowed to differentiate according to the rapid differentiation paradigm of Example 7 producing cultures enriched in neurons, astrocytes, and oligodendrocytes. Total RNA is extracted from the undifferentiated neurospheres as well as the differentiated cell cultures using the guanidinium thiocyanate acid phenol method referred to in Example 39. Messenger RNA (mRNA) is isolated by exploiting the affinity of its poly A tract to stretches of either U's or T's. Reverse transcription of the mRNA produced cDNA, is then used to make primary libraries in either plasmid [Rothstein et al., *Methods in Enzymology* 225:587–610 (1993)] or lambda phage vectors. To isolate cDNAs that are specific to either undifferentiated or differentiated stem cell derived progeny, cDNA from one is hybridized to RNA from the other, and vice versa. The unhybridized, and thus culture type-specific, cDNAs in each case are then used to construct subtracted libraries [Lopez-Fernandez and del Mazo, *Biotechnigues* 15(4):654–658 (1993)], or used to screen the primary libraries.

Stem cell-derived undifferentiated cell specific and differentiated cell specific cDNA libraries provide a source of

clones for novel markers and regulatory factors involved in CNS stem cell proliferation and differentiation. Specific cDNAs are studied by sequencing analysis to detect specific sequence motifs as clues to identity or function, and database searching for homologies to known transcripts. Using cDNAs in a hybridization to various RNA samples electrophoresed on an agarose-formaldehyde gel and transferred to a nylon membrane, allows the estimation of size, relative abundance, and specificity of transcripts. All or portions of cDNA sequences are used to screen other libraries in order to obtain either complete mRNA sequences or genomic sequence information. Antibodies directed against fusion proteins generated from specific cDNAs are used to detect proteins specific to a particular cell population, either by immunocytochemistry or by Western Blot analysis. Specific gene sequences are used to isolate proteins that interact with putative regulatory elements that control gene expression. These regulatory elements are then used to drive the expression of an exogenous gene, such as beta-galactosidase.

EXAMPLE 41: Electrophysiological Analysis of Neurons Generated From Growth Factor-Responsive Stem Cells and Exposed to a Biological Agent

Neurospheres were generated as described in Example 4. Neurospheres were dissociated using the technique described in paradigm 2, Example 7. The clonally derived cells were plated at low density and differentiated in the presence of bFGF. The electrophysiological properties of cells with the morphological appearance of neurons were determined as described as described by Vescovi et al. [*Neuron*, 11: 951–966 (1993)]. Under whole cell current clamp, the mean resting potential and input resistance were −62±9 mV and 372±MΩ. Rectangular suprathreshold current steps, (−100 pA) elicited regenerative potential responses in which the amplitude and time course were stimulus dependent. After the completion of electrophysiological experiments, the cell morphology was visualized by intracellular excitation of 5-carboxyfluorescein.

EXAMPLE 42: Screening for the Effects of Drugs or Other Biological Agents on Growth Factor-Responsive Stem Cell Progeny Generated From Tissue Obtained From a Patient with a Neurological Disorder

The effects of BDNF on the EGF-responsive stem cell progeny generated from CNS tissue obtained at biopsy from a patient with Huntington's disease is determined using the methods outlined in Example 7, A to E. BDNF is a potent differentiation factor for GABAergic neurons and promotes extensive neuronal outgrowth. Huntington's Disease is characterized by the loss of GABAergic neurons (amongst others) from the striatum.

EXAMPLE 43: Assay of striatum-derived neurosphere proliferation in response to various combinations of proliferative and regulatory factors

Paradigm 1: Primary striatal cells prepared as outlined in Example 1 were suspended in Complete Medium, without growth factors, plated in 96 well plates (Nunclon) and incubated as described in Example 4. Following a one hour incubation period, a specific proliferative factor, or a combination of proliferative factors including EGF, or bFGF (recombinant human bFGF: R & D Systems), or a combination of EGF and bFGF, or EGF plus FGF plus heparan sulfate (Sigma), or bFGF plus heparan sulfate made up in Complete Medium at a concentration of 20 ng/ml for each of the growth factors and 2 μg/ml for heparan sulfate), was added to each well of the plate.

Activin, BMP-2, TGF-β, IL-2, IL-6, IL-8, MIP-1α, MIP-1β, MIP-2 (all obtained from Chiron Corp.), TNFα, NGF (Sigma), PDGF (R&D Systems), EGF and CNTF (R. Dunn and P. Richardson, McGill University) were made up in separate flasks of compete medium to a final concentration of 0.2 μg/ml. Retinoic acid (Sigma) was added at a concentration of 10^{-6}M. 10 μl of one of these regulatory factor-containing solutions was added to each proliferative factor-containing well of the 96 well plates. Control wells, containing only proliferative factors, were also prepared.

In another set of experiments, the neurosphere inducing properties of each of these regulatory factors was tested by growing cells in their presence, in proliferative factor-free Complete Medium. None of these regulatory factors, with the exception of EGF, when used in the absence of a proliferation-inducing factor such as EGF or FGF, has an effect on neural stem cell proliferation.

The activin, BMP-2, TGF-μ. IL-2, IL-6, IL-8, MIP-1α, MIP-1β, MIP-2, TNFα and EGF additions were repeated every second day, CNTF which was added each day and retinoic acid, NGF and PDGF were added only once, at the beginning of the experiment. The cells were incubated for a period of 10–12 days. The number of neurospheres in each well was counted and the resulting counts tabulated using Cricket Graph III. Other relevant information regarding sphere size and shape were also noted.

In general, bFGF had a greater proliferative effect than EGF on the numbers of neurospheres generated per well. In the presence of 20 ng/ml EGF, approximately 29 neurospheres per well were generated. In the presence of bFGF, approximately 70 neurospheres were generated. However, in bFGF alone, the neurospheres were only about 20% of the size of those generated in the presence of EGF. The combination of EGF and bFGF produces significantly more neurospheres than does EGF alone, but fewer than seen with bFGF alone. The neurospheres are larger than those seen in bFGF alone, approximating those seen in EGF. In the case of bFGF generated spheres, the addition of heparan sulfate increased the size of the spheres to about 70% of the size of those which occur in response to EGF. These data suggest that EGF and FGF have different actions with respect to the induction of stem cell mitogenesis.

The effects of the regulatory factors added to the proliferative factor-containing wells are summarized in Table II. In general, the TGFβ family, interleukins, macrophage-inhibitory proteins, PDGF, TNFα, retinoic acid (10^{-6}M) and CNTF significantly reduced the numbers of neurospheres generated in all of the proliferative factors or combinations of proliferative factors tested. BMP-2 (at a dose of 10 ng/ml), completely abolished neurosphere proliferation in response to EGF. EGF and heparan sulfate both greatly increased the size of the neurospheres formed in response to bFGF (about 400%).

TABLE II

	PROLIFERATIVE FACTORS									
	EGF		bFGF		EGF + bFGF		bFGF + Heparan		EGF + bFGF + Heparan	
REGULATORY FACTORS	#	size	#	size	#	size	#	size	#	size
TGFβ Family♦	−57%	−	−57%	−	−34%	—	−55%	−	−20%	−
BMP-2	−100%	n/a	−5%	=	+16%	—	−3%	−	+10%	—
Interleukins	−21%	=	−23%	=	−37%	−	−28%	=	−39%	−
MIP Family	−25%	=	−6%	=	−32%	−	−22%	=	−33%	−
NGF	−10%	=	0%	=	−30%	=	+5%	=	−48%	=
PDGF	−1.5%	=	−4%	=	−26%	=	−10%	=	−27%	=
TNFα	−17%	=	−17%	=	−41%	=	−21%	=	−37%	=
10^{-6}M Retinoic Acid	−8%	—	−61%	−	−31%	—	−65%	—	−45%	—
CNTF	−23%	−	−77%	~	−81%	—	−81%	−	−84%	—
EGF	−		−14%	++	−		−17%	=	−	
Heparan Sulfate	0%	=	0%	++	0%	=				

♦Excluding BMP-2 (i.e. TGFα and activin)
Numbers of neurospheres generated (#) are given as percentages that reflect the decrease (−) or increase (+) in numbers of neurospheres per well, in response to a PROLIFERATIVE FACTOR in the presence of a REGULATORY FACTOR, compared with the number of neurospheres proliferated in the absence of the REGULATORY FACTOR.
Size of neurospheres generated in the presence of PROLIFERATIVE FACTORS and REGULATORY FACTORS compared to those generated in the presence of PROLIFERATIVE FACTORS alone are indicated as follows:
++: much larger; +: larger; =: approximately the same size; ~: variable in size; −: smaller; —: much smaller

Antisense and sense experiments were carried out using the following oligodeoxynucleotides (all sequences written 5' to 3'):

EGF receptor:	Sense strand:	GAGATGCGACCCTCAGGGAC (SEQ ID NO: 1)
	Antisense strand:	GTCCCTGAGGGTCGCATCTC (SEQ ID NO: 2)
EGF:	Sense strand:	TAAATAAAAGATGCCCTGG (SEQ ID NO: 3)
	Antisense strand:	CCAGGGCATCTTTTATTTA (SEQ ID NO: 4)

Each oligodeoxynucleotide was brought up and diluted in ddH_2O and kept at −20° C. Each well of the 96 well plates received 10 µl of oligodeoxynucleotide to give a final concentration of either 1, 2, 3, 4, 5, 10 or 25 µM. Oligodeoxynucleotides were added every 24 hours. The EGF receptor (EGFr) and EGF oligodeoxynucleotides were applied to cultures grown in bFGF (20 ng/ml), and EGFr oligodeoxynucleotides were applied to cultures grown in EGF (20 ng/ml). Cells were incubated at 37° C., in a 5% CO_2 100% humidity incubator. After a period of 10 to 12 days, the number of neurospheres per well was counted and tabulated. A concentration of 3 µM of antisense oligodeoxynucleotides produced a 50% reduction in the number of neurospheres generated per well, whereas the sense oligodeoxynucleotides had no effect on the number of neurospheres generated in response to EGF and FGF. Both the sense and antisense oligodeoxynucleotides were toxic to cells when 10 µM or higher concentrations were used.

Similar experiments can be performed using the following oligonucleotides:

FGF receptor:	Sense strand:	GAACTGGGATGTGGGGCTGG (SEQ ID NO: 5)
	Antisense strand:	CCAGCCCCACATCCCAGTTC (SEQ ID NO: 6)
FGF:	Sense strand:	GCCAGCGGCATCACCTCG (SEQ ID NO: 7)

-continued

Antisense strand:	CGAGGTGATGCCGCTGGC (SEQ ID NO: 8)

The FGF receptor (FGFr) and FGF oligodeoxynucleotides are applied to cultures grown in EGF, and FGFr oligodeoxynucleotides are applied to cultures grown in bFGF.

Paradigm 3: Embryonic tissue is prepared as outlined in Example 1 and plated into 96 well plates. Complete Medium, containing 20 ng/ml of either EGF of bFGF is added to each well. 10 µyl of diluted phorbol 12-myristate 13 acetate (PMA) is added once, at the beginning of the experiment, to each well of the 96 well plates, using an Eppendorf repeat pipetter with a 500 µl tip to give a final concentration of either 10, 20, 40, 100 or 200 µg/ml. Cells are incubated at 37° C. in a 5% CO_2 100% humidity incubator. After a period of 10 to 12 days the number of neurospheres per well is counted and tabulated.

Paradigm 4: Embryonic tissue is prepared as outlined in Example 1 and plated into 96 well plates. 10 µl of diluted staurosporine is added to each well of a 96 well plate, using an Eppendorf repeat pipetter with a 500 µl tip to give a final concentration of either 10, 1, 0.1, or 0.001 µM of staurosporine. Cells are incubated at 37° C., in a 5% CO_2 100% humidity incubator. After a period of 10 to 12 days, the number of neurospheres per well is counted and tabulated.

EXAMPLE 44: Adult spinal cord stem cell proliferation—in vitro responses to specific biological factors or combinations of factors

Spinal cord tissue was removed from 6 week to 6 month old mice, as follows: cervical tissue was removed from the vertebral column region rostral to the first rib; thoracic spinal tissue was obtained from the region caudal to the first rib and approximately 5 mm rostral to the last rib; lumbar-sacral tissue constituted the remainder of the spinal cord. The dissected tissue was washed in regular artificial cerebrospinal fluid (aCSF), chopped into small pieces and then placed into a spinner flask containing oxygenated aCSF with high Mg^{2+} and low Ca^{2+} and a trypsin/hyaluronidase and

kynurenic acid enzyme mix to facilitate dissociation of the tissue. The tissue was oxygenated, stirred and heated at 30° C. for 1½ hours, then transferred to a vial for treatment with a trypsin inhibitor in media solution (DMEM/12/hormone mix). The tissue was triturated 25–50 times with a fire narrow polished pipette. The dissociated cells were centrifuged at 400 r.p.m. for 5 minutes and then resuspended in fresh media solution. Cells were plated in 35 mm dishes (Costar) and allowed to settle. Most of the media was aspirated and fresh media was added. EGF alone, or EGF and bFGF were added to some of the dishes to give a final concentration of 20 ng/ml each, and bFGF (20 ng/ml) was added, together with 2 μg/ml of heparan sulfate, to the remainder of the dishes. The cells were incubated in 5% CO_2, 100% humidity, at 37° C. for 10–14 days. The numbers of neurospheres generated per well were counted and the results tabulated. EGF alone resulted in the generation of no neurospheres from any of the spinal cord regions. In the presence of EGF plus bFGF, neurospheres were generated from all regions of the spinal cord, in particular the lumbar sacral region. The combinations of EGF+FGF and FGF+ heparan sulfate produced similar numbers of spheres in the cervical region, whereas the combination of bFGF plus heparan sulfate resulted in fewer neurospheres from the thoracic and lumbar regions.

EXAMPLE 45: Transplantation of Multipotent Neural Stem Cell Progeny in Animal Models

I. TRANSPLANTATION PROCEDURE

1. Neurosphere preparation

Neural tissue was obtained from normal embryonic or adult CD1 mice and from embryonic or adult Rosa 26 mice (transgenic animals derived from C57/BL/6 mice, which express the β-galactosidase gene in all cells, thus allowing the transplanted cells to be easily detected in host tissue). Neurospheres were generated using the procedures described in Examples 1–5, passaged 2 to 8 times (see Example 6), and maintained in culture for 6–10 days after the last passage.

2. Labeling and Preparation of Neural Stem Cell Progeny

16 hours prior to transplantation, neurospheres derived from embryonic and adult tissue were labeled with BrdU by adding BrdU to the media for a total concentration of 1 μM and/or with fluorescent latex beads (Polysciences; 1:100 dilution of 0.75 μm beads). Neurospheres were detached from the substrate by gentle shaking, poured into 50 ml centrifuge tubes and spun down (5 minutes, 400 r.p.m., 15° C., no brake) to remove the proliferation-inducing media used for the proliferation culture. The neurospheres derived from embryonic tissue were then washed twice in Hank's buffered salt solution (HBSS), resuspended in 2 ml HBSS and dissociated by trituration (spheres drawn into a fire-polished pasteur pipette 40×). The neurospheres derived from adult tissue were trypsinized (0.05% in EDTA media; 5–10 min) and then a trypsin inhibitor (ovomucoid; 0.7–1.0 mg/ml in media) was added. The tubes were swirled and the neurospheres were recentrifuged (400 r.p.m.; 15° C., no brake). Cells were resuspended in 2 ml media (DMEM F12/hormone mix) and dissociated by mechanical trituration (25×).

Live and dead cells obtained from neurospheres derived from embryonic and adult tissue were counted prior to being centrifuged to remove dead cells (10 min., 400 r.p.m., 15° C., no brake). The live cells were resuspended to appropriate cell density (1–50×10^6 cells/ml). The cells were recounted to determine the number of live and dead cells and cell viability was calculated. The cells were then transferred to a microcentrifuge tube for storage on ice prior to transplantation. When ready for use, cells were resuspended prior to each cell injection by drawing cells into an eppendorf pipette tip (200 or 1000 μl).

3. Transplantation of Neural Stem Cell Progeny

The donor neural stem cell progeny were transplanted into selected sites in the brain of normal, healthy neonate or adult CD1 or C57BL/6 mice or adult Wistar or Sprague-Dawley rats. In some cases, embryonic cells from CD1 mice received in vitro gene transfer procedures prior to transplantation of the cells. The host animals were anaesthetized with sodium pentobarbital (65 mg/Kg) and placed into a stereotaxic apparatus. A skin incision was made to expose the surface of the skull or vertebrae. Injection sites were located using stereotaxic coordinates to locate the desired site. Burr holes were drilled in the skull and vertebrae at the coordinate sites. A 5 μl syringe was housed on a syringe pump and attached to a stainless steel cannula (30–31 gauge) via a short length of polyethylene tubing. A small air bubble and then 4–5 μl of the desired cell suspension was drawn into the cannula. The cannula was lowered to the desired location and 1–3 μl of the cell suspension was injected at a speed of 0.1–0.5 μl/min. Animals that received xenografts or allograft were treated with 0.1 mg/ml cyclosporin A in the drinking water to reduce the risk of tissue rejection.

4. Analysis of Transplanted Neural Stem Cell Progeny

The animals were allowed to survive for 2–12 weeks prior to sacrifice. At a specified time after transplantation, animals were perfused transcardially for aldehyde fixation of the brain and spinal cord tissue. A low-high pH perfusion protocol was used (Sloviter & Nilaver, (1987) *Brain Res.* Vol. 330:358–363). After perfusion, brains and spinal cords were removed, post-fixed, and then cryoprotected in sucrose/PBS for cutting in a cryostat. Sections of tissue (10 μM) were cut and mounted on microscope slides directly in a sequential way so that adjacent sections could be examined with different anatomical protocols.

Survival of transplanted cells labeled with fluorescent beads were identified by the localization of fluorescent beads within the cell cytoplasm. BrdU labeled cells (cells that had incorporated BrdU into their DNA during cell division in culture prior to transplantation) were identified using antibodies against BrdU (1:250–500; Monoclonal-Sera-lab; Polyclonal-Accurate Chem. & Sci). Antibodies against GFAP (1:250 Monoclona-Boehringer, Polyclonal-BTI), or NeuN (1:250–500; Monoclonal-R.J. Mullen) were then used to identify the differentiation of the transplanted cells. Cell transplants derived from transgenic animals expressing β-galactosidase were histochemically analyzed using methodology described by Turner and Cepko (1987) (*Nature* 328:131–136)and by immunohistochemical staining. For Rosa 26 cells, antibodies against β-galactosidase were used to identify the transplanted cells and antibodies to NeuN were used to identify cells that had differentiated into neurons. Human cells were identified with HLA antibodies (1:250, Monoclonal-Sera-labs). Antibodies were incubated with the tissue samples and detected using standard immunohistochemical protocols.

The results obtained from the animal models described below are summarized in Tables II–V.

A. MODEL OF HUNTINGTON'S DISEASE

Rats were anesthetized with nembutal (25 mg/kg i.p) and injected with atropine sulfate (2 mg/kg i.p.). Animals sustained an ibotenate lesion of the striatum, stimulating Huntington's Disease in the animals. 7 days after the lesion, the

animals received an injection of cells prepared as in Examples 1–5 under stereotaxic control. Injections were made to the lesioned area via a 21-gauge cannula fitted with a teflon catheter to a microinjector. Injected cells were labelled with fluorescein-abelled microspheres. Animals were given behavioral tests before the lesion, after the lesion, and at various intervals after the transplant to determine the functionality of the grafted cells at various post-operative time points, then killed and perfused transcardially with 4% buffered paraformaldehyde, 0.1% glutaraldehyde and 5% sucrose solution at 4° C. The brains were frozen in liquid nitrogen and stored at –20° C. until use. Brains sections were sliced to 26 μm on a cryostat, fixed in 4% paraformaldehyde and stained using the M6 monoclonal antibody to stain for mouse neurons, a nd then reacted with a secondary anti-rat fluorescein-conjugated antibody. Neuronal and glial phenotype was identified by dual labeling of the cells with antibody to NSE and GFAP.

B. PARKINSON'S DISEASE

Two animal models of Parkinson's Disease were used. In the first model, unilateral dopamine neurons of the substantia nigra were lesioned by the stereotaxic administration of 6-OHDA into the substantia nigra in adult CD 1 (1–4 μg) and C57BL/6 mice (1 μg), and Wistar rats (16 μg). Mice were pretreated with desipramine (25 mg/Kg i.p.) and rats were pretreated with pargyline (50 mg/Kg i.p.) both of which prevent the action of 6-OHDA on noradrenergic neurons and allow the selective destruction of dopaminergic neurons. In one series of experiments, multipotent neural stem cell proge ny obtained from embryonic Rosa 26 mice, were prepared using the procedures described in Examples 1 and 4. The neural stem cell progeny were labeled, prepared, and transplanted into the striatum of the lesioned C57BL/6 mice using the methods described above in this Example. In a second series of experiments, the cells were administered to the same regions in the brains of adult 6-OHDA Wister rats. In a third series of experiments, proliferated fetal human cells (prepared as outlined in Example 9), were transplanted into the striatum of the 6-OHDA lesioned CD1 mice. After a survival period of 2 weeks, the host animals were sacrificed and their brains removed. The brain tissue was treated and analyzed as described above. The second animal model used was the adult mutant Weaver mice (Jackson Labs, 3-½ months), in which approximately 70% of the dopaminergic neurons of the substantia nigra are lost by the age of 3 months. Animals were anaesthetized and the proliferated progeny of multipotent neural stem cells derived from embryonic Rosa 26 mice were injected into the striatal region of the animals according to the methods described above. The animals were allowed to survive for 15 days prior to sacrifice and analysis of striatal tissue.

C. CARDIAC ARREST

Transient forebrain ischemia was induced in adult Wistar rats by combining bilateral carotid occlusion with hypovolemic hypotension (Smith et al. (1984) *Acta Neurol Scand* 69:385–401). These procedures lesion the CA1 hippocampal pyramidal cells which is typical of damage observed in humans following cardiac arrest and the cause of severe memory and cognitive deficits. The progeny of proliferated multipotent neural stem cells, derived from embryonic Rosa 26 mice, were prepared as described above and transplanted into the lesioned hippocampal region of the ischemia lesioned rats. After 8 days, the animals were sacrificed and their brains were removed and analyzed. β-gal positive cells, indicating surviving cells from the Rosa 26 donor) were detected in the lesioned hippocampal region. In addition, double labeled β-gal/NeuN$^+$ cells were found indicating that transplanted cells had differentiated into neurons.

D. STROKE

Occlusion of the carotid arteries precipitates the occurrence of ischemic damage similar to that which occurs during stroke. Adult Wistar rats, in which the middle cerebral artery has been occluded to produce symptomatic lesions in the caudal striatum and parietal cortex, have neural stem cell progeny implanted into the lesioned areas. After a survival period, the animals are tested for behavioral improvements and are then sacrificed and their brains analyzed.

E. EPILEPSY

Implantation of an electrode into the amygdala is used to kindle the brain, inducing epileptic episodes and other symptoms of epilepsy. Neural stem cell progeny are transplanted into the hippocampal region. The animals are later tested for epileptic episodes and then sacrificed for analysis of the grafted tissue.

F. ALZHEIMER'S DISEASE

Cognitive impairment is induced in rats and mice by ibotenic acid lesions of the nucleus basalis, or old animals, exhibiting signs of dementia, are used. Neural stem cell progeny are transplanted into the frontal cortex, medial septal nucleus and the nucleus of the diagonal band of the brains of the animals. After a survival period, the animals are tested for cognitive ability and are then sacrificed to allow analysis of brain tissue.

G. SPINAL CORD INJURY AND DISEASE

Spasticity is a debilitating motor disorder that is a common consequence of disorders such as spinal cord injury, MS, and cerebral palsy. Transection of the spinal cord is used to produce muscular paralysis and is followed by the development of spasticity, which is characterized by debilitating hyperactive tendon reflexes, clonus and muscle spasms. Neural stem cell progeny are prepared and are transplanted into the lumbar lateral funiculus. After a survival period, the animals are examined for improvement in motor control and are then sacrificed to allow for analysis of spinal tissue.

TABLE III

DONOR CELL SOURCE	HOST	TRANSPLANT REGION	BrdU	BrdU/ GFAP	BrdU/ NeuN
Embryonic CD1 Mouse	Neonate	striatum	+	+	+
	CD1 Mouse	frontal cortex	+	+	+
	Adult CD1	striatum	+	+	+
	Mouse	hippocampus	+	+	+
		frontal cortex	+	+	+
		parietal cortex	+	+	+
		MS/NDB	+	+	+
	Adult Wistar	spinal cord	+	+	+
	Rat	hippocampus	+	+	+
		parietal cortex	+	+	
Adult CD1 Mouse	Adult CD1 Mouse	striatum	+	+	
		hippocampus	+		
		frontal cortex	+		
	Adult Wistar Rat	spinal cord	+		

TABLE IV

Donor Cell Source	HOST	Transplant Region	β-Gal	BrdU	BrdU/ GFAP
Embryonic CD1 Mouse	Adult CD1 Mouse	hippocampus	+	+	+
		frontal cortex	+	+	+

TABLE IV-continued

Donor Cell Source	HOST	Transplant Region	β-Gal	BrdU	BrdU/ GFAP
in vitro gene transfer		parietal cortex	+	+	+
		striatum	+	+	+
		MS/NDB	+	+	+
Embryonic Rosa	Adult DC1 Mouse	striatum	+	+	+
		parietal cortex	+	+	+
		MS/NDB	+	+	+
Adult Rosa 26	Adult C57/ BL/6 Mouse	hippocampus	+		
		frontal cortex	+		
		MS/NDB	+		

TABLE V

DONOR CELL SOURCE	HOST	β-Gal	BrdU/GFAP
Embryonic Rosa 26 Mouse	Adult 6-OHDA lesioned C57BL/6 mouse (striatal injections)	+	+
	Adult 6-OHDA lesioned Wistar rat (striatal injections)	+	+
Embryonic Rosa 26 Mouse	Adult Mutant Weaver Mouse (striatal injections)	+	+

All references, patents, and patent applications cited herein are incorporated herein by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 8

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAGATGCGAC CCTCAGGGAC 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTCCCTGAGG GTCGCATCTC 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TAAATAAAAG ATGCCCTGG 19

(2) INFORMATION FOR SEQ ID NO:4:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCAGGGCATC TTTTATTTA 19

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAACTGGGAT GTGGGGCTGG 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCAGCCCCAC ATCCCAGTTC 20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCCAGCGGCA TCACCTCG 18

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGAGGTGATG CCGCTGGC 18

What is claimed is:

1. A method of producing genetically modified neural cells comprising:

(a) obtaining cells derived from mammalian neural tissue containing at least one multipotent neural stem cell capable of producing progeny that are capable of differentiating into neurons, astrocytes, and oligodendrocytes, wherein said obtained cells have not been cultured in a serum-containing medium;

(b) preparing a substantially serum-free culture medium containing one or more predetermined growth factors capable of inducing multipotent neural stem cell proliferation, and

(c) preparing a cell culture by combining the cells obtained in (a) with the culture medium prepared in (b) to induce proliferation of said multipotent neural stem cell to produce proliferating multipotent neural stem cells and proliferating multipotent neural stem cell progeny which includes daughter multipotent neural stem cells and introducing exogenous DNA that does not contain oncogenes to genetically modify said proliferating multipotent neural stem cells and/or said multipotent neural stem cell progeny to produce genetically modified neural stem cell progeny.

2. The method of claim 1 wherein the growth factor in the culture medium prepared in (b) is selected from the group consisting of acidic fibroblast growth factor, basic fibroblast growth factor, epidermal growth factor, amphiregulin, transforming growth factor alpha, and combinations thereof.

3. The method of claim 1 wherein said growth factor in the culture medium prepared in (b) is epidermal growth factor or transforming growth factor alpha.

4. The method of claim 1 wherein said growth factor in the culture medium prepared in (b) is a fibroblast growth factor.

5. The method of claim 4 wherein said culture medium prepared in (b) additionally contains epidermal growth factor.

6. The method of claim 1 wherein the growth factor in the culture medium prepared in (b) is epidermal growth factor.

7. The method of claim 1 wherein the culture medium prepared in (b) is defined.

8. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to produce a growth factor product.

9. The method of claim 8 wherein said growth factor product is selected from the group consisting of nerve growth factor, brain-derived neurotrophic factor, neurotrophins, ciliary neuroatrophic factor, amphiregulin, basic fibroblast growth factor, acidic fibroblast growth factor, epidermal growth factor, transforming growth factor-alpha, transforming growth factor-beta, platelet-derived growth factor, insulin-like growth factors, and interleukins.

10. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to produce a neuropeptide.

11. The method of claim 10 wherein said neuropeptide is selected from the group consisting of substance-P, neuropeptide-γ, enkephalin, vasopressin, vasoactive intestinal peptide, cholecystokinin, glucagon, bombesin, somatostatin, and calcitonin gene-related peptide.

12. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to express a growth factor receptor.

13. The method of claim 12 wherein said growth factor receptor is selected from the group consisting of low affinity nerve growth factor receptor, ciliary neurotrophic factor receptor, neurotrophin receptors, epidermal growth factor receptor, fibroblast growth factor receptor, and amphiregulin receptor.

14. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to express a neurotransmitter.

15. The method of claim 14 wherein said neurotransmitter is selected from the group consisting of serotonin, L-dopa, dopamine, norepinephrine, epinephrine, tachykinin, endorphin, histamine, N-methyl D-aspartate, glycine, glutamate, γ-amino butyric acid, and acetylcholine.

16. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to contain a neurotransmitter synthesizing gene.

17. The method of claim 16 wherein said neurotransmitter synthesizing gene is selected from the group consisting of tyrosine hydroxylase, dopa decarboxylase, dopamine-β-hydroxylase, phenylethanolamine N-methyltransferase, glutamic acid decarboxylase, tryptophan hydroxylase, choline acetyltransferase, and histidine decarboxylase.

18. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to express a neurotransmitter receptor.

19. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to express chromaffin granule amine transporter.

20. The method of claim 1 wherein the multipotent neural stem cell progeny produced in (c) grow in the form of clonally-derived clusters of cells.

21. The method of claim 1 wherein prior to genetically modifying said proliferating multipotent neural stem cell progeny, a subsequent cell culture is prepared by combining said multipotent neural stem cell progeny with fresh substantially serum-free culture medium containing one or more predetermined growth factors which induces multipotent neural stem cell proliferation to proliferate said daughter multipotent neural stem cells to produce more progeny which include more daughter multipotent neural stem cells.

22. The method of claim 1 wherein said mammalian neural tissue is obtained from a juvenile or adult.

23. The method of claim 1 wherein said mammalian neural tissue is obtained from a human.

24. A method of producing genetically modified neural cells comprising:

(a) obtaining cells derived from juvenile or adult mammalian neural tissue containing at least one multipotent neural stem cell capable of producing progeny that are capable of differentiating into neurons, astrocytes, and oligodendrocytes;

(b) preparing a culture medium containing one or more predetermined growth factors capable of inducing multipotent neural stem cell proliferation; and

(c) preparing a cell culture by combining the cells obtained in (a) with the culture medium prepared in (b) to induce proliferation of said multipotent neural stem cell to produce multipotent neural stem cell progeny which includes daughter multipotent neural stem cells and introducing exogenous DNA that does not contain oncogenes to genetically modify said multipotent neural stem cell progeny.

25. A method of producing genetically modified neural cells comprising:

(a) obtaining cells derived from human neural tissue containing at least one multipotent neural stem cell capable of producing progeny that are capable of differentiating into neurons, astrocytes, and oligodendrocytes;

(b) preparing a culture medium containing one or more growth factors capable of inducing multipotent neural stem cell proliferation; and

(c) preparing a cell culture by combining the cells obtained in (a) with the culture medium prepared in (b) to induce proliferation of said multipotent neural stem cell to produce multipotent neural stem cell progeny which includes daughter multipotent neural stem cells

and introducing exogenous DNA that does not contain oncogenes to genetically modify said multipotent neural stem cell progeny.

26. The method of claim 1 further comprising:

(d) subjecting said genetically modified multipotent neural stem cell progeny to culture conditions that induce neural cell differentiation to produce a cell culture comprising genetically modified, differentiated neural cells selected from the group consisting of astrocytes, neurons, oligodendrocytes, and combinations thereof.

27. The method of claim 26 wherein in (d) the genetically modified multipotent neural stem cell progeny are differentiated in a culture medium containing serum.

28. The method of claim 26 wherein in (d) the genetically modified multipotent neural stem cell progeny are differentiated on a fixed substrate to which said multipotent neural stem cell progeny can adhere.

29. The method of claim 26 wherein in (d) the genetically modified multipotent neural stem cell progeny are differentiated in a culture medium containing a growth factor that influences differentiation.

30. A method of producing genetically modified differentiated neural cells comprising:

(a) obtaining cells derived from mammalian neural tissue containing at least one multipotent neural stem cell capable of producing progeny that are capable of differentiating into neurons, astrocytes, and oligodendrocytes, wherein said obtained cells have not been cultured in a serum-containing medium;

(b) preparing a substantially serum-free culture medium containing one or more predetermined growth factors capable of inducing multipotent neural stem cell proliferation,

(c) preparing a cell culture by combining the cells obtained in (a) with the culture medium prepared in (b) to induce proliferation of said multipotent neural stem cell to produce proliferating multipotent neural stem cells and multipotent neural stem cell progeny which includes daughter multipotent neural stem cells, and

(d) subjecting the cells proliferated in (c) to culture conditions that induce neural cell differentiation to produce differentiating and differentiated neural cells, and introducing exogenous DNA that does not contain oncogenes to genetically modify said differentiated and/or differentiating neural cells to produce genetically modified differentiated neural stem cell progeny.

31. A composition comprising genetically modified multipotent neural stem cell progeny prepared by the method of claim 1.

32. A composition according to claim 31 wherein said genetically modified multipotent neural stem cell progeny are genetically modified to express a biologically active substance selected from the group consisting of growth factor products, growth factor receptors, neurotransmitters, neurotransmitter receptors, neuropeptides, and neurotransmitter-synthesizing genes.

33. A composition comprising genetically modified differentiated multipotent neural stem cell progeny prepared by the method of claim 26.

34. A composition comprising genetically modified differentiated multipotent neural stem cell progeny prepared by the method of claim 30.

35. A composition comprising genetically modified differentiated neural cells produced by the method of claim 30.

36. A composition according to claim 35 wherein said genetically modified differentiated neural cells are genetically modified to express a biologically active substance selected from the group consisting of growth factor products, growth factor receptors, neurotransmitters, neurotransmitter receptors, neuropeptides, and neurotransmitter-synthesizing genes.

37. A composition comprising a population of non-primary neural cells which are derived from a primary cell culture, said population of non-primary neural cells having a greater percentage of multipotent neural stem cells compared to that of said primary culture, wherein a single multipotent neural stem cell is capable of producing progeny that are capable of differentiating into neurons, and glia, including astrocytes, and wherein said multipotent neural stem cells are genetically modified and do not contain exogenous oncogenes.

38. The composition of claim 37 wherein said percentage of multipotent neural stem cells of said population of non-primary neural cells is at least ten fold higher than that of said primary cell culture.

39. The composition of claim 37 wherein said multipotent neural stem cells are derived from human neural tissue.

40. The composition of claim 37 wherein said multipotent neural stem cells are derived from juvenile or adult neural tissue.

* * * * *

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Identifying Monoaminergic, GABAergic, and Cholinergic Characteristics in Immortalized Neuronal Cell Lines

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We measured the concentration of neurotransmitters in immortalized neural cell lines of hippocampal, septal, brainstem and cerebellar origin. While in most of the cell lines, concentrations of monoamines, γ-aminobutyric acid (GABA) and acetylcholine were low, in some they were markedly higher. This made it quite easy to identify possible monoaminergic, GABAergic or cholinergic cell lines. However all the cell lines contained glutamate and aspartate and there were no outstanding differences in levels of these amino acids differences between the cell lines. Deprivation of serum, which made the cells acquire a more differentiated morphology, caused an increase in the intracellular concentrations of some compounds and a switch from multiple to a single transmitter in the case of some cell lines. It suggested that measurement of transmitter concentrations combined with serum deprivation studies, may provide an indication of the neurochemical characteristics of immortalised neuronal cell lines.

KEY WORDS: Immortalized cell lines; amino acids; neurotransmitters; HPLC.

INTRODUCTION

Investigation of different neurobiological phenomena at the cellular and molecular level has so far been hindered by the heterogeneity, the low yield and the limited life span of primary cultures. To overcome these problems, a variety of immortalized neuronal cell lines have been generated in the past two decades by different laboratories. However, usually a limited number of cell lines of different characteristics were involved when their neurochemical characterisation was performed. Also, it is hard to compare results of one laboratory with another because of the different processing protocol of the cells.

In this laboratory, clonal neuronal cell lines of brainstem and cerebellar origin were established by recombinant retrovirus-mediated transduction of v-myc (1) oncogene, or by somatic cell fusion (2). Morphological and immunocytochemical characteristics of the cell lines were described in detail previously (3–5). It is worth noting that when differentiated by standard methods (retinoic acid and/or serum deprivation), they all expressed 68 kD, 160 kD and 200 kD neurofilaments, microtubule associated protein-2 and neuron specific enolase antigens, all of which are characteristic for neuronal phenotypes. The hybrid cell lines expressed these antigens even in the presence of serum without any differentiating agents (4,5).

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Multiple neurotransmitter phenotypes have been reported in neuroblastoma cell lines (6–8) and a wide variety of neurotransmitters were found to be co-localized in mature single neurons of various regions of the central nervous system (9–13). These observations suggest that a single neuron may have multiple sets of structural genes which encode a variety of neurotransmitter-producing and metabolising machinery. Since some of the cell lines established in this laboratory showed immunoreactivity to multiple neurotransmitter antibodies (4,5), it was difficult to judge if these cell lines were really multipotential or specific to a certain phenotype.

In the present study, in order to get information about neurochemical characteristics of these cell lines, we systematically measured their neurotransmitter content, and compared them with other reference cell lines and primary cultures. We also studied the effect of serum deprivation on the transmitter levels in all the cell lines.

EXPERIMENTAL PROCEDURE

Cell Lines. The following cell lines were used in this study. PC12 (14) is a pheochromocytoma line with well characterized catecholamine secreting properties. SN6.1b (5) and SN6.2a (5) are subclones of the mouse septal cholinergic hybrid cell line SN6.10.2.2 (2). Several cell lines were generated by somatic cell fusion of N18TG2 (15), a hypoxanthine phosphoribosyltransferase-deficient mouse neuroblastoma line, with cerebellar primary culture of new-born (CL5a4-1, CL8c4-7 (4), CL8a5-2 (4)), 6 day-old (CL10a5-3), 7 day-old (CL7d1-4), 10 day-old (CL12a1-2, CL12c2-1) and 28 day-old (M1b1-2, M1e1-1) mice.

HV16-1 (3), HV16-2, HV16-3, HV16-4 (3) were generated from hippocampi of 14 day-old rat embryos by recombinant retrovirus-mediated transduction of v-myc oncogene.

Septal, hippocampal and hindbrain primary cultures, that were made according to Kamegai et al. (16) from the brain of 15 day-old mouse embryos, the non-secreting neuroblastoma line (N18TG2), C6 glioma line (17), C2C12 myoblastoma line (18) and a myelin basic protein-specific nonencephalitogenic T cell line (4b14a/n) were used as reference.

Measurement of Protein, Amino Acid, Acetylcholine (ACh), and Monoamine Concentrations. The cells were cultured until confluency in Dulbecco's modified Eagle's medium containing 100 U/ml penicillin and 100 μg/ml streptomycin supplemented with 10% foetal calf serum (FCS) or not (serum-free medium) then they were mechanically harvested. Aliquots were taken for the measurement of protein, amino acid, ACh, and monoamine concentrations. If not indicated, data were accumulated from five independent experiments.

The protein content of the cells was measured according to Lowry's method, after lysing the cells overnight in 1% sodium dodecylsulfate and separating DNA by spinning at 100,000 *g* for 1 h at 20°C. Bovine serum albumin was used as standard.

For the measurement of amino acids, the cells were homogenised in 3% sulfosalicylic acid, the precipitated protein was pelleted by spinning at 25,000 *g* for 20 min at 2°C. The supernatant was passed through a 0.22 μm filter and was applied to a Hitachi L-8500 amino acid analyser. Concentrations of Asp, Asn, Glu, Gln, Gly and GABA were determined by using fluorescent detection after post-column derivatization with o-phtalaldehyde. Results were expressed as nmol/mg protein.

ACh and choline were measured by using an HPLC method (19). Results were expressed as nmol/mg protein.

After removal of proteins by perchloric acid precipitation, we measured the monoamines, norepinephrine (NE), epinephrine, dopamine (DA) and serotonin (5-HT); their precursors, dihydroxy phenylalanine (DOPA) and 5-hydroxy tryptophan (5-HTP); and their metabolites, dihydroxyphenylethyleneglycol, 3-methoxy-4-hydroxyphenyl glycol, normetanephrine (NMN), metanephrine, 3,4-dihydroxyphenylacetic acid, 3-O-methyldopa (MDOPA), homovanillic acid (HVA), vanillic acid, 3-methoxytyramine (3-MT) and 5-hydroxyindolacetic acid by using Neurochem (Niko Bioscience, Tokyo), a new gradient HPLC system with a 16-coulometric electrode-array detector (20). Results were expressed as ng/mg protein.

Statistical analysis was performed by using ANOVA followed by Dunett's test for multiple comparisons.

Release Experiments. For release experiments, SN6.1b and HV16-4 cells were cultured until confluency in 10 cm plastic dishes in serum containing medium. HV16-4 cells, but not SN6.1b cells, were induced to differentiate by exposing them for three days to serum-free medium containing 1 mM retinoic acid. For determination of spontaneous and depolarisation-induced efflux of the compounds studied, culture medium was aspirated, cells were washed twice with phosphate buffered saline and incubated for 5 min at 37°C in 5 ml of physiological salt solution (containing in mM: NaCl 135, $CaCl_2$ 1, $MgCl_2$ 0.75, glucose 5, HEPES 10 pH 7.4) in the presence of 5 and 26 or 56 mM of KCl respectively. To keep osmolarity of the solutions when elevated KCl concentration was used, the concentration of NaCl was reduced to 109 or 79 mM respectively. Amino acid, ACh and monoamine concentrations were determined from the 5 min incubating mediums and from the cells after the experiments. In similar experiments, $CaCl_2$ was omitted and 1 mM of EDTA was present in the 5 min incubating mediums. Data were expressed as fractional rate/mg protein of the cells.

Statistical analysis was performed by using paired t-test.

RESULTS

In order to determine their neurochemical characteristics, we systematically measured the concentration of transmitters in all the cell lines and in reference cells. We found that the immortalised hippocampal and cerebellar cell lines, as well as the primary cultures of matching age, did not contain significant amounts of monoamines (Table I, column 3–5). It made it relatively easy to identify cell lines that may use monoamines as a functional transmitter. Interestingly the SN6 lines, which are generally considered to be cholinergic (2), also contained the monoamines; NE, DA and 5-HT (Table I, column 3–5).

Results with ACh were qualitatively the same as with monoamines. Most of the cell lines were devoid of this compound, while the amounts of ACh found in

Table I. Neurotransmitter Levels in the Cell Lines

Cell lines	Compounds						
	ACh	NE	DA	5-HT	GABA	Asp	Glu
	pmol/mg protein	ng/mg protein			nmol/mg protein		
PC12	370.1	2.4	46.8	n.d.	0.1	6.4	28.8
SN6.10.2.2	110.2	n.d.	4.6	22.9	0.3	13.0	42.5
SN6.1b	479.2	2.3	5.1	32.7	0.4	16.6	52.6
SN6.2a	79.0	0.5	4.2	7.1	0.3	6.2	18.5
CL5a4-1	n.d.	n.d.	n.d.	n.d.	0.8	10.8	17.6
CL8c4-7	n.d.	n.d.	0.1	0.3	0.3	13.9	37.5
CL8a5-2	n.d.	n.d.	n.d.	1.7	1.0	12.1	37.5
CL10a5-3	n.d.	n.d.	n.d.	n.d.	0.4	7.6	21.5
CL7d1-4	n.d.	n.d.	n.d.	n.d.	0.2	11.2	27.9
CL12a1-2	n.d.	n.d.	n.d.	n.d.	0.5	17.2	40.4
CL12c2-1	n.d.	n.d.	n.d.	n.d.	0.3	10.7	34.9
M1b1-2	n.d.	n.d.	n.d.	0.6	0.5	5.1	17.4
M1e1-1	n.d.	0.7	n.d.	0.1	1.0	12.3	42.3
HV16-1	133.1	n.d.	n.d.	n.d.	0.9	3.7	18.8
HV16-2	n.d.	n.d.	n.d.	n.d.	0.2	2.4	10.2
HV16-3	n.d.	n.d.	n.d.	n.d.	1.2	6.0	38.1
HV16-4	n.d.	n.d.	n.d.	n.d.	5.8	3.2	13.5
Septal	80.1	n.d.	n.d.	n.d.	2.9	6.9	16.8
Hippocampal	150.0	n.d.	n.d.	n.d.	1.6	3.6	9.3
Hindbrain	300.2	n.d.	n.d.	n.d.	2.9	4.8	12.0
N18TG2	n.d.	n.d.	n.d.	n.d.	0.3	9.2	16.9
C6	n.d.	n.d.	n.d.	n.d.	0.2	4.5	9.5
C2C12	n.d.	n.d.	n.d.	n.d.	n.d.	1.1	1.5
4b14a/n	n.d.	n.d.	n.d.	0.5	1.1	24.7	15.3

Septal, hippocampal and hindbrain primary cultures were cultured in serum-free medium, while the other cell lines in serum-containing medium. Values represent the mean of five independent experiments. SEM values (not shown) were less than 15% of the mean values. Contents below detection limit are indicated by n.d.

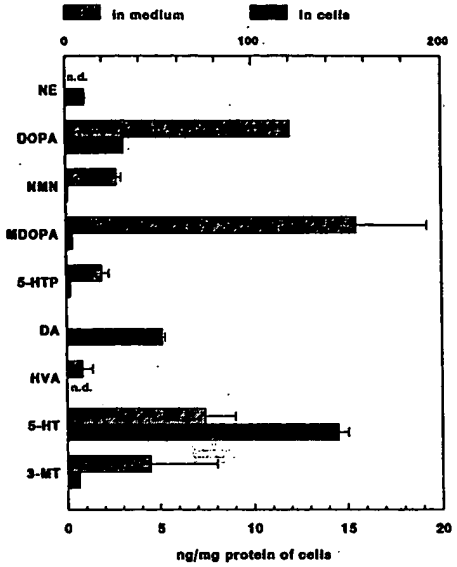

Fig. 1 Comparison of monoamine-related compounds of SN6.10.2.2 cells and of the medium. After culturing for three days, cells and samples of medium were processed for HPLC analysis (see Methods). Results are expressed as ng/mg protein of the cells. Scale for intracellular contents (top y axis) is 10 times the scale for extracellular contents (bottom y axis) indicating the marked difference between the two pools. S.E.M., coming from one experiment of three dishes running in parallel, is indicated by error-flag. Contents below detection limit are indicated by n.d.

PC12, HV16-1 and SN6 lines were similar to those of corresponding primary cultures (Table I, column 2).

All the cell lines contained some GABA, but the GABA content of HV16-4 line was higher than that of primary cultures (Table I, column 6). Since primary cultures represent a heterogeneous population of cells, where the level of a given transmitter should be lower than in a homogeneous population on the per mg protein basis, we considered just this cell line as possibly GABAergic.

In contrast to monoamine transmitters, it was very difficult to identify cell lines of glutamatergic phenotype. In the case of mature neurons, the level of Glu clearly indicates whether this compound has transmitter or metabolic function (21). Although some of the cell lines contained higher amounts of Glu than the others (Table I, column 8), it was hard to characterise them as glutamatergic. This was because there did not seem to be any critical threshold value since Glu concentrations were continuously distributed in the range of 1.5–52.6 nmol/mg protein.

The same held true for Asp, because the T cell line, which was expected to be completely devoid of neurotransmitters, contained the highest level of Asp (Table I, column 7).

We also determined the pattern of neurotransmitter-related compounds and their distribution between culture medium and the cells. SN6.10.2.2 cell line was chosen to demonstrate the results of these experiments, because this cell line contained several monoamine-related compounds. As shown in Fig. 1, the metabolites were mostly present in the culture medium, while monoamine transmitters were found mostly intracellularly, except for 5-HT. As compared to the pattern of monoamine-related compounds in brain tissue, a common feature was found in all the monoamine containing cell lines. DOPA and MDOPA concentrations, which are normally hardly detectable in the brain, were very high (Fig. 1).

We tested the effect of serum deprivation on the levels of transmitter-related compounds in all the cell lines. Results of cell lines with neurochemical characteristics distinguishing them from the rest, as well as reference cell lines, are presented in Table II. Generally, serum deprivation caused a decrease in the concentrations of all the compounds except for ACh and GABA. GABA levels increased by 46% in HV16-4 cell line, and

Table II. Effect of Serum Deprivation on the Cell Lines

	Compounds						
	ACh	NE	DA	5-HT	GABA	Asp	Glu
Cell lines	pmol/mg protein		ng/mg protein		nmol/mg protein		
PC12	180.2	n.d.	5.3	n.d.	n.d.	3.1	7.8
	49%***	0%***	11%***	n.p.	0%***	48%*	27%***
SN6.10.2.2	145.2	n.d.	n.d.	n.d.	0.2	6.6	25.2
	132%	n.p.	0%***	0%***	65%	51%	59%*
SN6.1b	608.6	n.d.	n.d.	n.d.	n.d.	10.3	40.0
	127%	0%***	0%***	0%***	0%***	62%*	76%
SN6.2a	105.1	n.d.	n.d.	n.d.	0.1	5.3	15.9
	133%	0%***	0%***	0%***	37%**	85%	86%
HV16-1	215.6	n.d.	n.d.	n.d.	0.6	2.1	11.8
	162%**	n.p.	n.p.	n.p.	71%	57%*	63%*
HV16-4	n.d.	n.d.	n.d.	n.d.	8.5	3.7	9.9
	n.p.	n.p.	n.p.	n.p.	146%*	116%	74%
N18TG2	180.7	n.d.	n.d.	n.d.	n.d.	7.2	15.5
	n.p.***	n.p.	n.p.	n.p.	0%***	79%	92%
C6	n.d.	n.d.	n.d.	n.d.	0.2	4.5	6.4
	n.p.	n.p.	n.p.	n.p.	90%	58%**	67%*
C2C12	n.d.	n.d.	n.d.	n.d.	n.d.	1.3	1.7
	n.p.	n.p.	n.p.	n.p.	n.p.	121%	111%
4b14a/n	n.d.	n.d.	n.d.	n.d.	n.d.	9.1	12.1
	n.p.	n.p.	n.p.	0%***	0%***	37%**	79%

Concentrations of compounds in the cells cultured in serum-free medium are expressed as the mean of two independent experiments of three dishes running in parallel in each (upper row) and as means % (lower row) of the concentrations in the cells cultured in serum-containing medium. SEM values (not shown) were less than 15% of the mean values. Any significant difference, $p < 0.05$, $p < 0.01$ and $p < 0.001$ from the values obtained when the cells were cultured in serum-containing medium is indicated by *, ** and *** respectively. Contents below detection limit are indicated by n.d. Absence of a given compound in the cells when cultured in serum-containing medium is indicated by n.p.

yet decreased or were lacking in other cell lines. ACh decreased in the PC12 line, increased by 30% in the SN6 lines and increased significantly in HV16-1 and N18TG2 lines. In the SN6 lines, there was a complete disappearance of the monoamines.

In order to test which transmitter is functional in SN6.1b, we performed "static" release experiments in the presence and absence of Ca^{2+}. There was a relatively high rate of outflow of the compounds measured that correlated with the Na^+-rather than with K^+-concentration of the medium (data not shown). Efflux of the compounds was slightly higher in the absence than in the presence of Ca^{2+} (data not shown). We tested the GABA release from HV16-4 with qualitatively the same result (data not shown).

DISCUSSION

There is a good correlation between transmitter concentration or turnover and the activity of synthesising enzyme in the brain both anatomically (22,23) and during ontogenesis (24), but some reports have raised questions about this correlation in the case of cell lines (25,26). That is why we used transmitter concentrations rather than the presence or activity of enzymes as a first screen for neurochemical characterisation of immortalised cell lines.

Another problem with cell lines is that because of repeated replatings, the cells become synchronised. It is worth noting that while amino acids decreased, the other compounds increased to a maximum then gradually decreased with longer culture times (data not shown). To deal with this problem, we measured the concentrations of compounds of interest from three dishes running in parallel on five different occasions. Data in Table I represents average values of these measurements to allow better comparison of cell lines. However, data in Figure 1 and Table II is from one and two experiments respectively of three dishes running in parallel.

Since we had powerful analytical instruments, we measured the transmitters together with related compounds from the same sample (see Methods). This had the advantage that from the metabolite pattern we could identify aberrant transmitter metabolism in the cells. Normally, DOPA level is very low in brain tissue (27),

because tyrosine hydroxylase is the rate-limiting enzyme in the production of DA. Under pathophysiological conditions, when activity of aromatic amino acid decarboxylase (AADC) is diminished, DOPA levels increase and a normally lacking metabolite, MDOPA appears (28) because of the action of catechol-O-methyltransferase on DOPA. In PC12 cell line (data not shown) as well as in SN6 cell lines, DOPA content was high and there was a substantial amount of MDOPA (Fig. 1), suggesting a decrease in activity and/or expression for AADC in these cell lines.

Fig. 1 shows that the metabolites were present mostly in the culture medium while all the transmitters except for 5-HT, were found mostly inside the cells. As it turned out, 5-HT was not a real exception, since about 3.9 μg/ml 5-HT was found in FCS that could account for the 5-HT content of the medium. However, since a low level of metabolites could be detected intracellularly (Fig. 1), we measured just the intracellular pool of the transmitters and transmitter-related compounds.

We measured about 60 times less DA in PC12 cell line as Greene and Tischler (14). Also they found DA/NE to be 2.7, while it was 19.5 in our case. These differences could arise from deviation of our PC12 cell line from the original. We chose the non-neuronal PC12 line as a reference cell lines for studying catecholaminergic properties, since (i) it contained a lot of NE, DA, all DA metabolites and all the enzymes concerning catecholamine metabolism (14) (ii) and upon serum deprivation it responded in a non-neuronal way. (In catecholaminergic neuronal cell lines, deprivation of serum increased catecholamine content (29,31)).

The hippocampal and cerebellar cell lines did not contain monoamines, indicating that the immortalisation did not drastically change the expression of transmitter phenotype in these cell lines. However considerable amounts of monoamines were found in the SN6 cell lines. The monoamine levels that we measured in SN6 cell lines were much lower than those in human neuroblastoma clone IMR32 (29), ten times lower than in a DAergic cell line MES23.5 (30), and were of the same order as in mouse teratocarcinoma 1C11 (31).

The presence of multiple transmitters in neuroblastomas is quite common (7,29), but the original SN6 cell line was characterised to be cholinergic (2). In agreement with this view, the disappearance of monoamines in serum-free medium in SN6 cells (Table II) suggested that monoamines could not play a transmitter role in SN6 cell lines, since in DAergic clones, deprivation of serum caused a 2 to 3.5-fold increase in DA content (29,31).

Beside SN6 cell lines, hippocampal line HV16-1 was characterised as possibly cholinergic (Table I.). ACh concentrations in these cell lines were about in the same range as in septal or hippocampal primary cultures (Table I) although 2 to 10 times less than that of the SN56.B5.G4 (32) cell line. Also, ACh in these cell lines increased by 30–60% in serum-free medium (Table II). Serum deprivation caused an appearance of ACh in the so called non-secreting line N18TG2. It is worth noting that several lines of neuroblastoma C-1300, from which N18TG2 originated, is cholinergic (15). However, serum deprivation did not cause any morphological changes in the N18TG2 line (data not shown).

Based upon the same argument we used for identifying cholinergic properties, we characterised the hippocampal line HV16-4 as possibly GABAergic (Table I). The GABA level found in this cell line was about the same as in primary cultures and in NG108-15 line (25) and it increased by 40% in serum-free medium (Table II).

We could not identify glutamatergic or aspartatergic cell lines because of two reasons: (i) We did not have appropriate positive controls and (ii) concentrations of these amino acids were quite evenly distributed between the lowest and the highest value (Table I). Asp and Glu did not increase significantly in response to serum deprivation in any of the cell lines tested (Table II).

Previous reports (6,7,29,31–33) as well as the results of serum-deprivation experiments suggested that similarly as in the brain, in cell lines, a more mature phenotype is concomitant with a switch from multiple to a single transmitter and/or with an increase of transmitter content (Table II). However, in release experiments we found that the immortalised cell lines did not show the Ca^{2+}-dependent depolarisation-evoked transmitter release, which is a functional characteristic of mature neurons. All these results suggested that these cell lines represented an early stage of neuronal differentiation when cells are committed to neuronal phenotype but fail to express all the characteristics of a fully mature neuron.

There were several cell lines without any clear neurochemical characteristics. Some of them may have potentiality for a transmitter phenotype that we did not test in this study e.g. peptidergic. Another possibility is that they were so called non-secreting or inactive lines like several clones of neuroblastoma C-1300 (15). It is worth noting that these non-secreting lines could represent neuronal cells of such an early stage of development (34) that expression of their neurotransmitter phenotype was below detection limit. Sometimes they may have unex-

pected features like the presence of nitric oxide synthase in the N18TG2 cell line (35).

Neurochemical characterisation of immortalized cell lines is more difficult than in the brain. In the adult brain tissue, neurons are fully mature and establish synapses with other cells, so their nerve endings contain a lot of synaptic vesicles with a high transmitter content. Also, during development, there is a large increase (up to some hundred times) in either transmitter content or synthesising enzyme activity (24). The cell lines consist of one type of cells, so they hardly establish functional synapses. Their processes contact each other but they rarely develop processes with synaptic vesicles. Except for a few cell lines (PC12, NG108-15 (36) and SN56.B5.G4 (32)), Ca^{2+}-dependent transmitter release cannot be evoked from clonal cell lines and so one cannot verify whether the suggested transmitter is really functional or not. Also, upon induced differentiation, there is just a 1.5 to 6 times increase in either the transmitter content or synthesising enzyme activity (7,29,32,33).

Despite these difficulties, our results suggest that the measurement of transmitter concentrations combined with serum deprivation studies may provide an indication of the neurochemical characteristics of immortalised neuronal cell lines.

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Effect of Mutant α-Synuclein on Dopamine Homeostasis in a New Human Mesencephalic Cell Line*

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Mutations in *α-synuclein* have been linked to rare, autosomal dominant forms of Parkinson's disease. Despite its ubiquitous expression, mutant α-synuclein primarily leads to the loss of dopamine-producing neurons in the substantia nigra. α-Synuclein is a presynaptic nerve terminal protein of unknown function, although several studies suggest it is important for synaptic plasticity and maintenance. The present study utilized a new human mesencephalic cell line, MESC2.10, to study the effect of A53T mutant α-synuclein on dopamine homeostasis. In addition to expressing markers of mature dopamine neurons, differentiated MESC2.10 cells are electrically active, produce dopamine, and express wild-type human α-synuclein. Lentivirus-induced overexpression of A53T mutant α-synuclein in differentiated MESC2.10 cells resulted in down-regulation of the vesicular dopamine transporter (VMAT2), decreased potassium-induced and increased amphetamine-induced dopamine release, enhanced cytoplasmic dopamine immunofluorescence, and increased intracellular levels of superoxide. These results suggest that mutant α-synuclein leads to an impairment in vesicular dopamine storage and consequent accumulation of dopamine in the cytosol, a pathogenic mechanism that underlies the toxicity of the psychostimulant amphetamine and the parkinsonian neurotoxin 1-methyl-4-phenylpyridinium. Interestingly, cells expressing A53T mutant α-synuclein were resistant to amphetamine-induced toxicity. Because extravesicular, cytoplasmic dopamine can be easily oxidized into reactive oxygen species and other toxic metabolites, mutations in α-synuclein might lead to Parkinson's disease by triggering protracted, low grade dopamine toxicity resulting in terminal degeneration and ultimately cell death.

The main pathological hallmarks of Parkinson's disease (PD)[1] are a striking loss of dopamine (DA)-producing neurons in the substantia nigra, causing reduced DA levels in the striatum, and the presence of cytoplasmic inclusions known as Lewy bodies (1, 2). Even though oxidative stress and mitochondrial dysfunction have been implicated in the disease process, the mechanisms underlying nigral cell death in PD are still unknown (3). Most cases of PD are sporadic, but rare, familial forms of the disease do exist. To date, early-onset PD has been linked to mutations in two genes, *α-synuclein* and *parkin* (4). Autosomal dominant forms of the disease result from missense mutations in *α-synuclein*, leading to either an alanine to threonine substitution at position 53 (A53T) (5) or to an alanine to proline conversion at amino acid 30 (A30P) (6). Although these mutations are not present in the majority of patients with familial PD, pathogenic mechanisms involved in α-synuclein-mediated DA cell loss may provide important clues about sporadic and familial forms of the disease.

First identified as a component of cholinergic vesicles in the electric ray *Torpedo californica* (7), α-synuclein is a highly conserved, "natively unfolded," 140-amino acid phosphoprotein belonging to a family of closely related members (8). The function of α-synuclein is still unknown, although several studies suggest it plays an important role in synapse maturation and maintenance. α-Synuclein is enriched in presynaptic terminals (9–11) and is expressed rather ubiquitously in the brain, particularly in the neocortex, hippocampus, striatum, thalamus, and cerebellum (12). Its expression is developmentally regulated, redistributing from neuronal cell bodies to synaptic terminals during periods of neuronal differentiation (13, 14). Its expression is up-regulated during periods of synaptic plasticity, *i.e.* song-learning in the zebra finch (10). Lastly, overexpression of A53T mutant human α-synuclein in mice results in massive axonal degeneration of spinal cord motor neurons (15–17).

If α-synuclein plays an essential role in synaptic function, why do its mutant forms primarily lead to the degeneration of nigral DA neurons? This phenomenon could be explained by the cytotoxic potential of DA, a neurotransmitter that readily auto-oxidizes in the presence of iron and can also be metabolically deaminated to yield toxic DA metabolites and reactive

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[1] The abbreviations used are: PD, Parkinson's disease; DA, dopamine; ROS, reactive oxygen species; TH, tyrosine hydroxylase; DAT, dopamine transporter; VMAT2, vesicular monoamine transporter 2; AMPH, D-amphetamine; WT, wild-type; A53TSYN, A53T mutant human α-synuclein; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; PBS, phosphate-buffered saline; EGF, epidermal growth factor; CMV, cytomegalovirus; PDL, poly-D-lysine; db-cAMP, dibutyryl cyclic AMP; GDNF, glial cell line-derived neurotrophic factor; PVDF, polyvinylidene difluoride; SOD, superoxide dismutase; HPLC-EC, high-performance liquid chromatography coupled to electrochemical detection; GFP, green fluorescent protein; DHE, dihydroethidium; ANOVA, analysis of variance; EPSP, excitatory postsynaptic potential; IPSP, inhibitory postsynaptic potential; m.o.i., multiplicity of infection; 6-OHDA, 6-hydroxydopamine; PLD2, phospholipase D isoform 2.

oxygen species (ROS), *e.g.* superoxide anions and hydroxyl radicals (18–20). Thus, a failure to properly store DA into synaptic vesicles may lead to abnormal elevations of cytosolic DA followed by generation of cytotoxic DA metabolites and ROS. This could, in turn, lead to oxidative stress, terminal degeneration, and eventually cell death.

The present study examined the possibility that expression of A53T mutant α-synuclein leads to DA cell loss by promoting DA-dependent oxidative stress, a mechanism that has been proposed to underlie the toxicity of both amphetamine and 1-methyl-4-phenylpyridinium (MPP^+) (21–23). To test the hypothesis that mutant α-synuclein alters DA homeostasis, we first characterized a new human mesencephalic cell line, MESC2.10, and determined it to be a useful model system for studying the function of α-synuclein. In addition to expressing biochemical markers of mature DA neurons, MESC2.10 cells are electrically active, synthesize and release DA, and express wild-type (WT) human α-synuclein. Interestingly, lentiviral-induced expression of A53T mutant in MESC2.10 cells led to changes in DAT and VMAT2 protein levels, DA uptake and release, cytoplasmic DA immunofluorescence, intracellular superoxide production, formation of α-synuclein-positive inclusions, and response to amphetamine (AMPH)-induced toxicity, modifications that suggest alterations in DA function.

EXPERIMENTAL PROCEDURES

Generation of MESII(1)C2.10 Cells—Human mesencephalic cells were prepared from 8-week-old human embryonic ventral mesencephalic tissue (Lund University), which was procured in compliance with national laws and regulations, following permission from the Lund University Hospital Ethical Committee. Tissue fragments were maintained at 4 °C for ~72 h in Hanks' balanced salt solution (HBSS) without calcium and magnesium prior to mechanical dissociation in the presence of 3 mg/ml protease 23. Dispersed cells were then rinsed with HBSS containing 1 mg/ml trypsin inhibitor and 1 mg/ml bovine serum albumin prior to plating. Cells were seeded at a density of 5×10^4 cells/cm^2 in growth medium consisting of DMEM/F-12 and 10% fetal calf serum. Approximately 24 h later, the growth medium was replaced with DMEM/F-12 containing N2 supplement (Invitrogen, Gaithersburg, MD), 40 ng/ml human recombinant basic fibroblast growth factor (bFGF, Roche Molecular Biochemicals, Indianapolis, IN), 40 ng/ml human recombinant epidermal growth factor (EGF, Invitrogen), and 20 ng/ml platelet-derived growth factor A/B (PDGF, Roche Molecular Biochemicals, Indianapolis, IN).

Human mesencephalic cells were immortalized with a LINX v-myc retroviral vector (24). In this system, a tetracycline-controlled transactivator strongly activates transcription from a minimal CMV promoter, which, in turn, drives v-myc expression in the absence of tetracycline. A gene conferring neomycin resistance is also present in the vector allowing selection of v-myc-expressing cells. Mesencephalic cultures were retrovirally infected and G418-selected as previously described (25). After selection, cultures were maintained in N2 medium consisting of DMEM/F-12 high glucose, N2 supplement, 2 mM L-glutamine, 40 ng/ml bFGF, 40 ng/ml EGF, and 20 ng/ml PDGF. Confluent cultures were passaged by trypsin digestion. Clonal cell lines were isolated by two rounds of limited dilution in 96-well plates. Single colonies were expanded and passaged.

Differentiation of Human Mesencephalic Cells—MESC2.10 cells were propagated in N2 medium containing 40 ng/ml bFGF. After reaching confluency, cells were enzymatically dissociated with trypsin followed by trituration. Poly-D-lysine (PDL) pre-coated labware was treated overnight with 5 μg/ml mouse laminin (dissolved in PBS with Ca^{2+}) and washed three times prior to plating. Cells were seeded at a density of 2.5×10^4 cells/well in 8-well chamber slides, 7.5×10^4 cells/well in 24-well plates, or 2.0×10^5 cells/well in 12-well plates depending on the assay conducted. One day after plating, proliferation medium was replaced with N2 medium containing 1 μg/ml tetracycline, 1 mM dibutyryl cyclic AMP (db-cAMP), and 2 ng/ml glial cell line-derived neurotrophic factor (GDNF, R&D Systems, Minneapolis, MN), heretofore called differentiation medium. Half of this medium was replaced every second day. Clone MESC2.10 was chosen for further characterization, because a high percentage of cells from this line expressed both microtubule-associated protein 2 and tyrosine hydroxylase (TH).

Western Immunoblotting—MESC2.10 cells were plated at a density of 1×10^6 cells per PDL/laminin-coated P100 dish, allowed to become confluent, and differentiated for 0, 2, 4, or 6 days. Day 0 cells were lysed the same day that differentiation medium was added to prospective day 2–6 dishes. Briefly, cells were rinsed with ice-cold PBS and lysed in hypotonic buffer containing 2 mM EDTA, pH 7.6, 2 mM Hepes, 12 mM *N*-ethylmaleimide, and protease inhibitors (Sigma mixture). Cells were homogenized by passing them five to six times through a 27-gauge needle. After centrifuging at $800 \times g$ for 10 min at 4 °C, the supernatant was collected and SDS was added to 1% final concentration. An equal volume of 50 μg of protein (as measured with a Bio-Rad DC protein assay) from day 0–6 samples was heated for 10 min at 55 °C in the presence of Laemmli buffer containing 4% β-mercaptoethanol. Proteins were separated on a 10–20% continuous gradient SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane. Blots were blocked in 5% nonfat milk/PBS with 0.05% Tween 20 and incubated overnight at 4 °C in blocking buffer containing the following primary antibodies: mouse anti-rat TH (1:1000, Chemicon International, Temecula, CA), mouse anti-human β-tubulin isotype III (1:2000, Sigma), mouse anti-human α-synuclein (1:100, Alexis Biochemicals, San Diego, CA), rabbit anti-human vesicular monoamine transporter 2 (VMAT2, 1:500, kind gift from Dr. Robert Edwards), rabbit anti-human DA transporter (DAT before dilution, 1:1000, Alpha Diagnostic International), rabbit anti-cow glial fibrillary acidic protein (before dilution 1:2000, DAKO A/S, Glostrup, Denmark), and rabbit anti-human myc (1:1000, Upstate Biotechnology Inc., Lake Placid, NY). After washing 3× for 10 min in PBS/Tween 20, blots were incubated with 1:5000 horseradish peroxidase-linked secondary antibodies (from donkey, in blocking buffer) recognizing mouse or rabbit species followed by detection using an ECL Plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ). Day 4 MESC2.10 cells transduced for 48 h with LV-GFP or LV-A53TSYN were lysed as previously described, and immunoblots were probed with antibodies against α-synuclein, DAT, VMAT2, and TH, rabbit anti-human Cu,Zn-superoxide dismutase (SOD, 1:3000, StressGen Biotechnology Corp., Victoria, British Columbia, Canada), and mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (1:300, Chemicon International). Band intensity was quantified using IMAGE software (National Institutes of Health, Bethesda, MD).

Electrophysiology—Electrical activity was measured using the perforated whole-cell configuration of the patch clamp technique, using an EPC-9 amplifier and Pulse software (version 8.50 or later, Heka Elektronik, Lambrecht, Germany). Recording electrodes were made from borosilicate glass capillaries coated with Sylgard close to the tips and fire-polished. The pipette resistance ranged between 3 and 6 megohms when filled with the intracellular solution specified above. The zero-current potential of the pipette was adjusted in the bath prior to gigaseal formation. Cells were continuously superfused with extracellular solution consisting of (in mM) 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 25 D-glucose, and 5 Hepes (pH 7.4 with NaOH) at 32–34 °C. The pipette solution contained (in mM) 76 K_2SO_4, 10 NaCl, 10 KCl, 1 $MgCl_2$, and 5 HEPES (pH 7.35 with KOH), and 0.24 mg/ml of the pore-forming antibiotic amphotericin B (Sigma Chemical Co., St. Louis, MO).

Measurement of DA Content and Release by HPLC-EC—MESC2.10 cells were plated at a density of 2×10^5/well in PDL/laminin-coated 12-well plates. After 24 h, cells were exposed to differentiation medium for 0–6 days, lysed in 200 μl of 0.1 N perchloric acid containing 0.8 mg/ml glutathione by freeze/thawing, and the supernatants were collected for analysis of intracellular DA levels. Samples from day 0, 2, 4, and 6 cultures were frozen at −80 °C and analyzed by high-performance liquid chromatography coupled to electrochemical detection (HPLC-EC). For assays of DA release, day 6 MESC2.10 cells were treated with 60 mM KCl or 50 μM AMPH for 20 min at 37 °C in 300 μl of HBSS containing Ca^{2+} and 0.8 mg/ml glutathione. The extracellular medium was collected and filtered through a 0.2-μm filter. A sample volume of 30 μl was injected into an ESA Coulochem II electrochemical detector using a YMCaqua C18 column (Schermbeck, Germany) set at a potential of 300 mV *versus* an Ag/AgCl reference electrode. The mobile phase was delivered by an LC 10 AD Shimadzu HPLC pump at 0.5 ml/min and contained 0.051 M $NaH_2PO_4 \cdot H_2O$ with 0.92 mM octanesulfonic acid, 48 μM Na_2EDTA, and 11% methanol, adjusted to pH 3.7 with 1 M phosphoric acid and degassed. Data was acquired using a Shimadzu (chromatographic CLASS LC-10) software package. The analyses were calibrated to a standard of 0.2 μM DA. Assays were conducted in triplicate and experiments repeated three times.

Construction of Lentiviral Vectors—Mutant (A53T) human *α-synuclein* (A53TSYN) was isolated from an adeno-associated virus, rAAV-A53TSYN (kindly provided by Nina Rosenqvist, Lund University, Lund, Sweden), by PCR amplification using the following oligonucleotides: 5′-GTC AAG ATC TAT GGA TGT ATT CAT GAA AGG

ACT-3′ and 5′-AGT CCT CGA GTT AGG CTT CAG GTT CGT AGT-3′. The pHR′CMV-A53TSYN-WHV construct was generated by ligating this PCR product with a lentiviral backbone obtained by excising GFP from pHR′CMV-GFP-WHV (kindly provided by Dr. Didier Trono, University of Geneva Medical School, Geneva, Switzerland) using *Bam*HI and *Xho*I. These restriction sites were introduced into the flanking sequences of A53TSYN by PCR. The construct was sequenced at the Center for Genomics Research, Karolinska Institute (Stockholm, Sweden) to ensure that no PCR-based mutations were generated.

Virus Production—Lentiviral vectors were produced as previously described (26, 27). Briefly, 293T cells were transiently transfected by a calcium phosphatase method with a three-plasmid vector system consisting of: pHR′CMV-GFP-WHV or pHR′CMV-A53TSYN-WHR (transducing vector containing the transgene), pMD.G (vector encoding the envelope protein), and pCMVΔR8.91 (packaging construct providing all viral proteins needed except for the envelope). All constructs were kindly provided by Dr. D. Trono. The supernatant was collected 2 and 3 days after transfection and ultracentrifuged at 141,000 × *g* for 1.5 h. The pellet was resuspended in DMEM/10% FCS and frozen at −80 °C. The virus titer was determined by infecting 293T cells with serial dilutions of virus and counting the number of GFP or α-synuclein-positive cells (as assessed by immunocytochemistry). Untransduced 293T cells did not express α-synuclein. The dilution resulting in <30% of GFP or α-synuclein-positive cells was used to calculate transducing units/cell. A multiplicity of infection (m.o.i.) = 10, corresponding to 10 transducing units/cell, was used at all times to infect MESC2.10 cells.

[^{3}H]DA Uptake and Release—MESC2.10 cells were plated at a density of 7.5 × 10^4 cells/well in PDL/laminin-coated 24-well plates and transduced for 48 h with LV-GFP or LV-A53TSYN starting on day 4. On day 6, cells were rinsed with HBSS containing Ca^{2+} and incubated with 4.8 μCi/ml [7,8-^{3}H]DA/HBSS + Ca^{2+} (Amersham Biosciences, Piscataway, NJ) for 20 min at 37 °C. To measure intracellular [^{3}H]DA, the supernatant was removed and cells were lysed with 0.1 N perchloric acid by freeze/thawing. To measure basal [^{3}H]DA release, [^{3}H]DA-loaded cells were rinsed extensively with HBSS, and the supernatant was analyzed. For stimulation-induced [^{3}H]DA release, cells were treated with 60 mM KCl or 50 μM AMPH for 20 min at 37 °C and extracellular [^{3}H]DA measured. All samples were analyzed with a 1214 Rackbeta liquid scintillation counter (LKB Wallac, Turku, Finland). Experiments were conducted in quadruplicate and repeated three times.

Immunocytochemistry—For DA antibody experiments, day 4 MESC2.10 cells were transduced with LV-GFP or LV-A53TSYN for 48 h and exposed on day 6 to 50 μM AMPH for 20, 40, or 60 min at 37 °C. After fixation with 4% paraformaldehyde, cells were rinsed in PBS and preincubated with 5% normal goat serum/0.3% Triton-X/PBS for 1 h at room temperature. The following primary antibodies (in Triton X-100/PBS) were then added overnight at 4 °C: mouse anti-human β-tubulin isotype III (1:2000, Sigma, St. Louis, MO), rabbit anti-rat TH (1:100, Pel-Freeze, Rogers, AR), mouse anti-human α-synuclein (1:1000, Zymed Laboratories, San Francisco, CA), rabbit anti-DAT (1:500, Alpha Diagnostic International, San Antonio, TX), and mouse anti-DA (1:500, Fitzgerald, Concord, MA). After rinsing 3× in PBS, cells were incubated in either goat anti-rabbit Alexa 594 or goat anti-mouse Alexa 488 for 1 h at room temperature, rinsed in PBS, and cover-slipped in PVA-DABCO. In some cases, cells were incubated in 1 μg/ml Hoechst 33258 during the second to last wash before mounting. For assessment of α-synuclein-positive inclusions, day 6 MESC2.10 cells overexpressing GFP or A53TSYN were stained with a mouse anti-human α-synuclein antibody (see above). Cells were examined with a Bio-Rad MRC1024 confocal microscope using a 100× objective. For DA antibody experiments, the mean cytoplasmic florescence intensity from ≥50 cells per experiment was quantified using Bio-Rad LaserSharp software (Hercules, CA).

Dihydroethidium Imaging—Production of ROS was monitored in MESC2.10 cells overexpressing GFP or A53TSYN exposed to AMPH using the superoxide-sensitive fluorophore dihydroethidium (DHE) as previously described (22, 23, 28). MESC2.10 cells were plated at a density of 2.5 × 10^4 cells/well in PDL/laminin-coated eight-well chamber slides and differentiated for 4 days. Cells were then transduced with LV-GFP or LV-A53TSYN for 48 h. On day 6 transduced MESC2.10 cells were exposed to 50 μM AMPH for 60 min or 2 h followed by 20-min incubation with 3 μM DHE at 37 °C. Cells were then fixed with 4% paraformal dehyde and mounted. Fluorescence was measured at excitation = 488 nm and emission = 590 nm on a Bio-Rad MRC1024 confocal microscope using a 60× objective. The mean cytoplasmic fluorescence from ≥50 cells/experiment was quantified using LaserSharp software. Experiments were repeated three to five times.

Determination of Cell Viability with Calcein-AM—MESC2.10 cells were plated at a density of 2.5 × 10^4 cells/well in PDL/laminin-coated eight-well chamber slides and differentiated for 3 days. On day 3, cells were transduced with LV-GFP or LV-A53TSYN for 24 h and then exposed to 10, 50, or 100 μM AMPH for 48 h. On day 6, cells were incubated with 5 μM Calcein-AM for 20 min at 37 °C. Five consecutive images per well were captured with a BMK 800 digital camera (Grundig Electronic, Nürnberg, Germany) using a 10× objective. Calcein-AM-positive cells were counted and normalized to LV-GFP control cultures. Experiments were conducted in duplicate and repeated three to five times.

Statistical Analysis—All parameters are expressed as means ± S.E. of three to five independent experiments, each treatment performed in three to four wells. The significance of effects between untreated and treated, or between LV-GFP- and LV-A53TSYN-expressing, cells, was determined by one- or two-way factor analysis of variance (ANOVA), as indicated, and post-hoc Dunnett's *t* test using a Statview package (Abacus Concepts, Berkeley, CA).

RESULTS

Differentiated MESC2.10 Express Markers of Mature DA Neurons—Eight-week-old human embryonic mesencephalic cells were immortalized with a retroviral vector containing the v-*myc* oncogene (Fig. 1) (29). In brief, the control elements of a tetracycline-resistance operon were fused to a human CMV promoter directing the expression of v-myc (24). In this system, addition of low, non-toxic concentrations of tetracycline abolish transcription activation by a tetracycline-controlled transactivator thus blocking v-myc expression (29). Constitutive expression of v-myc in the absence of tetracycline enabled MESC2.10 cells to proliferate continuously in culture. Addition of tetracycline in the presence of GDNF, a factor that promotes the survival and morphological differentiation of DA neurons (30), and dibutyryl cyclic AMP (db-cAMP), a known inducer of TH activity (31), arrested proliferation and promoted the differentiation of MESC2.10 cells. After 6 days in differentiation medium, a high percentage of MESC2.10 cells expressed both the microtubule-associated protein 2 and the rate-limiting enzyme in dopamine biosynthesis, tyrosine hydroxylase (TH) (29). Therefore, this clone was chosen for further characterization.

To determine which cell types were present in differentiated MESC2.10 cultures, the expression of various neuronal, astrocytic, and DA markers was assessed by Western immunoblotting and immunocytochemistry. Because microtubules play an essential role in neurite outgrowth, synapse formation, and axonal and dendritic transport, β-tubulin is often used as a marker of immature and mature neurons (31, 32). Immunoreactivity for β-tubulin isotype III was faintly present in lysates from undifferentiated MESC2.10 cultures and was up-regulated when cells were exposed to differentiation medium, reaching robust levels at days 4 and 6 (Fig. 2*A*). Similarly, day 6 cultures immunoprocessed with an antibody against β-III-tubulin exhibited long, branched processes characteristic of mature neurons. Double staining with the nuclear marker Hoechst 33258 showed that at least 80% of Day 6 cells were neurons (Fig. 2*B*). On the other hand, the astrocytic marker glial fibrillary acidic protein was modestly expressed in undifferentiated MESC2.10 cultures and was down-regulated in response to differentiation (Fig. 2*A*). Like β-III-tubulin, expression of α-synuclein was barely detected at day 0 by Western immunoblotting but increased markedly by day 6. This antigen was also readily detected by immunocytochemistry and was localized to the cell body and to a lesser extent to neurites (see Fig. 5*C*).

To assess the presence of a DA phenotype, blots were also probed for antigens expressed in mature DA neurons. Although completely absent in undifferentiated cells, TH was highly expressed at days 4 and 6. Double immunocytochemistry for TH and Hoechst 33258 revealed that >80% of day 6 MESC2.10 cells were TH-positive (see Fig. 4*A*). TH was expressed both in the cell soma and neuritic processes. Like TH, the plasma

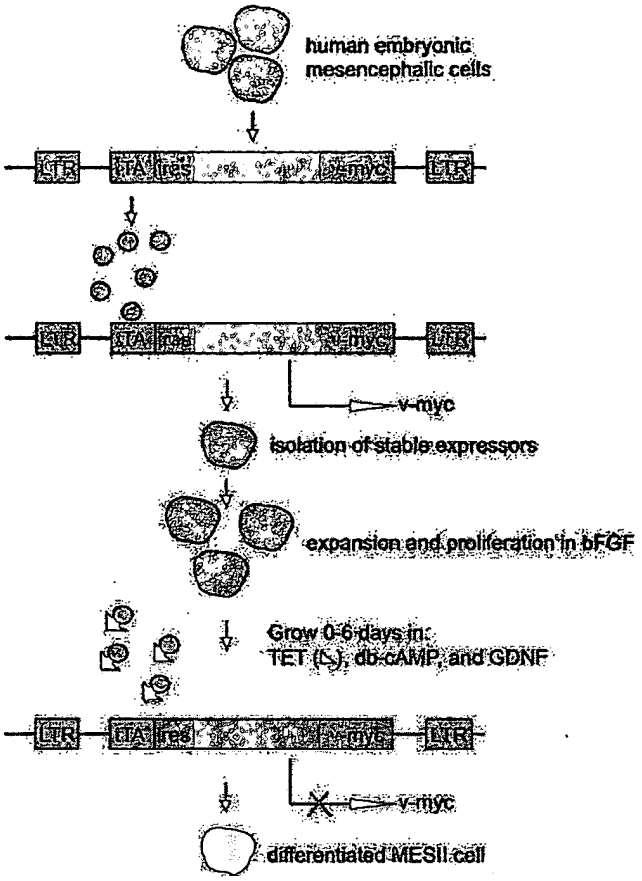

FIG. 1. **Generation of MESC2.10 cells.** First trimester human mesencephalic cells were dissociated and plated at a density of 5×10^4 cells/cm^2 in growth medium consisting of DMEM/F-12 and 10% fetal calf serum. Approximately 24 h later, the growth medium was replaced (see "Experimental Procedures") and cells retrovirally infected with a LINX v-*myc* vector. In this system, a tetracycline-controlled transactivator (*tTA*, *yellow circles*) strongly activates transcription from a minimal CMV promoter in the absence of tetracycline. A gene conferring neomycin resistance is also present in the vector. Cells expressing myc were G418-selected in N2 medium consisting of DMEM/F-12 high glucose, N2 supplement, 2 mM L-glutamine, 40 ng/ml bFGF, 40 ng/ml EGF, and 20 ng/ml PDGF. Clonal cell lines were isolated by two rounds of limited dilution, and single colonies were expanded and passaged. Constitutive expression of v-myc in the absence of tetracycline allowed MESC2.10 cells to proliferate continuously in culture. Replacement with N2 medium containing 1 μg/ml tetracycline (*purple triangle*) abolished transcription activation by tTA and blocked the production of v-myc. In addition to tetracycline, N2 medium also contained 1 mM db-cAMP and 2 ng/ml GDNF, factors that promoted the differentiation of MESC2.10 cells into DA-producing neurons (see "Results").

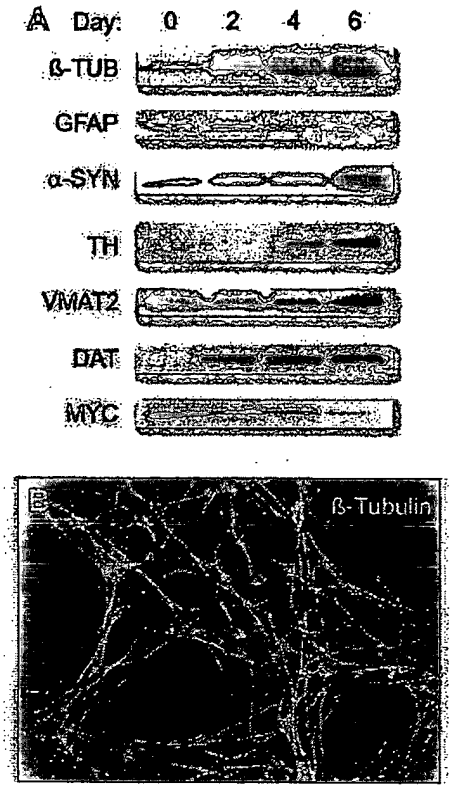

FIG. 2. **Differentiated MESC2.10 cultures display markers of mature DA neurons.** *A*, MESC2.10 cells were differentiated for 0–6 days in the absence of bFGF and in the presence of tetracycline, db-cAMP, and GDNF. Cultures were then lysed, and the expression of various proteins was assayed by Western immunoblotting. Briefly, equal amounts of protein were separated in 10–20% continuous gradient SDS-PAGE gels, transferred onto polyvinylidene difluoride membranes, and probed with antibodies against neuronal, astrocytic, synaptic, DA, and proliferative markers as listed under "Experimental Procedures." Western blots for each marker were repeated three to five times. *B*, MESC2.10 cultures that had been differentiated for 6 days were fixed and immunoprocessed with an antibody recognizing anti-human β-tubulin isotype III (shown in *green*), a marker of immature and mature neurons. Cultures were co-stained with the nuclear dye Hoechst 33258 (shown in *blue*). Cells were examined by confocal microscopy using a 20× objective.

membrane-bound DAT was absent in lysates from undifferentiated cultures but was present at day 2, 2 days before TH expression could be detected. Its robust expression did not change by day 6. Morphologically, DAT expression resembled the uniform pattern of TH expression (Fig. 4*B*). In contrast, expression of the vesicular monoamine transporter (VMAT2), which mediates vesicular storage of DA, was modestly expressed at day 0 and did not change in response to differentiation. As expected, tetracycline suppressed transcription activation of the CMV promoter and inhibited the production of myc, which became barely detectable at day 6 (Fig. 2*A*).

MESC2.10 Cells Are Electrically Active—The presence of neuronal (*e.g.* β-III-tubulin) and synaptic (*e.g.* α-synuclein) markers in Day 6 cultures suggested that differentiated MESC2.10 cells behave as functional neurons. To assess the electrophysiological properties of these cells, we applied the perforated whole-cell configuration of the patch-clamp technique to obtain membrane potential recordings from Day 6 MESC2.10 cells. 6 out of 16 studied cells showed spontaneous action potentials (Fig. 3*A*) that originated from a resting potential of -59 ± 2 mV and peaked at -12 ± 4 mV. The majority of the recorded cells (10 of 16) further displayed upward deflections from the resting potential consistent with excitatory postsynaptic potentials (EPSPs) and some (4 of 16) cells had inhibitory postsynaptic potentials (IPSPs) as well (Fig. 3*B*), indicating functional synapses. In several cases, EPSPs occurred close enough in time for summation (Fig. 3*C*).

MESC2.10 Cells Synthesize and Release DA upon Stimulation—To determine whether MESC2.10 cultures synthesize DA, intracellular DA levels were measured by HPLC-EC. Briefly, day 0–6 cultures were lysed with 0.1 N perchloric acid/0.8 mg/ml glutathione by freeze/thawing, and DA levels were analyzed by HPLC-EC. Intracellular DA content increased progressively in response to differentiation and was 9-fold higher in day 6 MESC2.10 cultures than in day 0 cultures (Fig. 4*C*). Our calculations revealed intracellular DA content to be 16.1 ± 0.1 pmol/TH-positive cell at day 6. We then tested whether day 6 MESC2.10 cells could release DA in response to high potassium stimulation. Exposure to 60 mM KCl for 20 min promoted

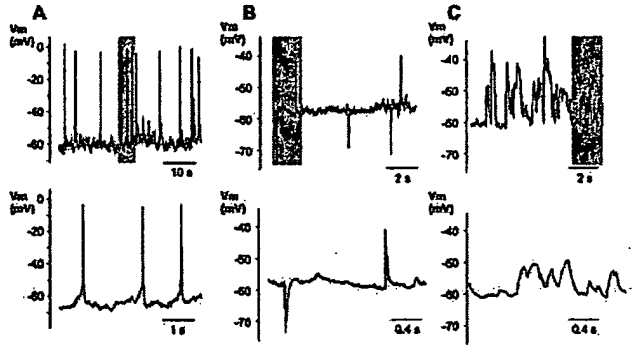

FIG. 3. **MESC2.10 cells are electrically active.** *A–C*, recordings of the membrane potential obtained in the perforated whole-cell configuration. *A*, typical action potentials; *B*, well resolved excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs); *C*, an example of the summation of EPSPs. The *lower panels* show the segments highlighted in *gray* on an expanded time scale.

an 8-fold increase in extracellular DA as measured by HPLC-EC, suggesting that the exocytic machinery of DA-synthesizing MESC2.10 cells is fully functional (Fig. 4*D*). This was also corroborated by patch-clamp capacitance measurements (data not shown). In addition to displaying exocytic release, differentiated MESC2.10 cells also extruded DA by reverse transport. The weak base psychostimulant AMPH has a high affinity for the DAT and is actively transported into DA neurons where it triggers the non-exocytic release of DA (33–36). Within the cell, AMPH blocks DA transport into vesicles by dissipating the transmembrane pH gradient, which provides the driving force for VMAT2-mediated uptake of DA (37). This leads to the accumulation of cytoplasmic DA that is either newly synthesized or has been taken up from the extracellular space. An increase in cytosolic DA concentration, in turn, promotes reverse transport of the neurotransmitter through the DAT, a phenomenon known as DA overflow (28, 38, 39). Therefore, AMPH can be used to measure non-exocytic, transporter-mediated DA release. Exposure of day 6 MESC2.10 cells to 50 μM AMPH for 20 min elicited a 9-fold increase in DA release, suggesting that both DAT-mediated uptake and release in MESC2.10 cells is functionally intact (Fig. 4*D*).

Lentiviral-induced Overexpression of A53T Mutant α-Synuclein Leads to Changes in DA Homeostasis—The use of HIV-based recombinant retroviruses has proven to be a highly effective method for transferring foreign DNA into non-dividing and terminally differentiated cells. Due to its karyophilic properties, the lentiviral pre-integration complex allows viral sequences to be readily recognized by the nuclear transport machinery (40). Lentiviral vectors mediate efficient delivery, integration, and sustained long term expression into post-mitotic cells such as adult neurons (41, 42). In addition, deletion of ≥60% of the viral genome, including all non-essential accessory proteins, has dramatically increased the biosafety of these retroviruses (43).

The goal of this study was to use a lentiviral transduction system to introduce A53TSYN into differentiated MESC2.10 cells to examine the effect of the mutant protein on intracellular DA homeostasis. The efficiency of lentiviral transfer was first tested by transducing undifferentiated and differentiated MESC2.10 cells with a lentivirus expressing the reporter green fluorescent protein (LV-GFP). Increasing dilutions of virus were used to determine the minimum m.o.i. needed to transduce >80% of MESC2.10 cells. Although LV-GFP used at an m.o.i. = 10 effectively transduced >80% of day 6 MESC2.10 cells, it did not infect undifferentiated MESC2.10 cells. The majority of LV-GFP-transduced cells showed high, uniform

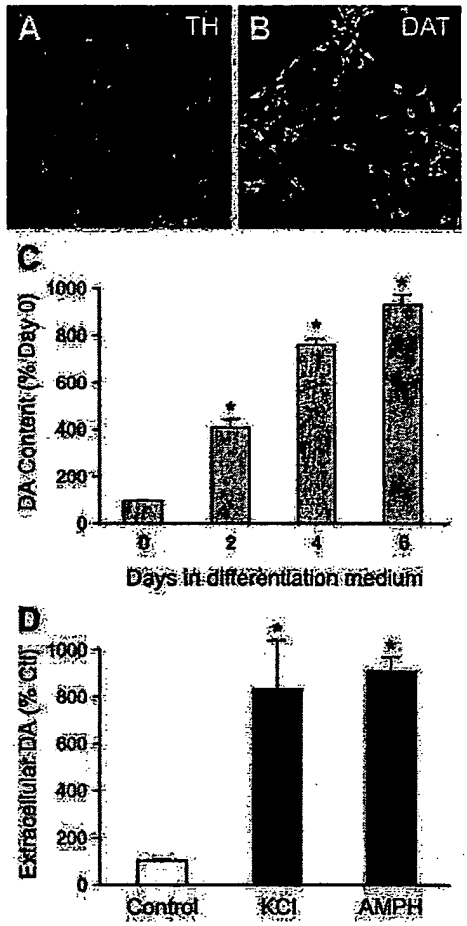

FIG. 4. **Differentiated MESC2.10 cells synthesize and release DA.** *A*, MESC2.10 cultures were differentiated for 6 days, fixed, and immunoprocessed with a rabbit antibody against TH (shown in *red*). Cells were co-stained with Hoechst 33258 to visualize nuclei, which appear *pink* due to co-localization with TH. *B*, Day 6 cultures were stained with a rabbit antibody recognizing DAT. Confocal images were taken with a 20× objective. *C*, MESC2.10 cultures differentiated for 0–6 days were lysed in 0.1 N perchloric with glutathione by freeze/thawing and the extracellular fluid collected for HPLC-EC analysis of DA. Values are expressed as a percentage of day 0. *D*, Day 6 MESC2.10 cells were exposed to 60 mM KCl or 50 μM AMPH in HBSS/Ca^{2+} for 20 min at 37 °C, and the levels of extracellular DA were measured by HPLC-EC. Values are expressed as percent increase over control. All experiments were conducted in quadruplicate. The *graphs* represent the mean ± S.E. of three independent experiments. *, $p < 0.01$ compared with day 0 (one-way ANOVA with post-hoc Dunnett's *t* test).

expression of GFP (*arrowhead*, Fig. 5*A*), although some displayed a more punctate pattern of fluorescence (*arrow*, Fig. 5*A*). Even though GFP was detected both in the cell body and in cellular processes resembling axons, a few cells expressed GFP primarily in the cell soma.

Having established that a lentiviral strategy can be used effectively to introduce foreign genes into differentiated MESC2.10 cells, a lentiviral vector containing human A53TSYN (LV-A53TSYN) was generated. In this study, we chose to concentrate on the effects of A53TSYN expression on DA homeostasis in human mesencephalic neurons. It can be argued that overexpression of WT human α-synuclein on MESC2.10 cells would have provided unequivocal proof that the effects seen in this study were indeed due to a pathogenic effect of the mutant protein and not to the overexpression of α-synuclein itself. In addition, it would have been helpful to compare all parameters in mutant α-synuclein-expressing cells, untransduced cells, and cells infected with an empty

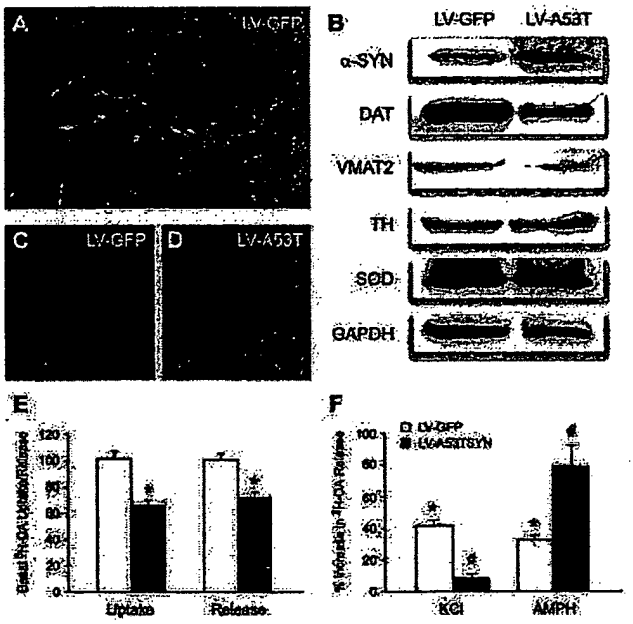

FIG. 5. **Lentivirus-mediated overexpression of A53T mutant α-synuclein.** *A*, day 6 MESC2.10 cells were transduced with a lentivirus overexpressing GFP for 48 h. Endogenous GFP fluorescence was visualized using a 20× objective. Cells with strong, uniform GFP expression (*arrowhead*) and cells with lesser, granular expression (*arrow*) can be observed. *B*, Day 4 MESC2.10 cultures were transduced with LV-GFP or LV-A53TSYN for 48 h and lysed, and the expression of different markers was assessed by Western immunoblotting. Briefly, proteins were separated in 10–20% continuous gradient SDS-PAGE gels, transferred onto polyvinylidene difluoride membranes, and probed with antibodies against α-synuclein, DAT, VMAT2, and TH; Cu,Zn-SOD; and glyceraldehyde-3-phosphate dehydrogenase, which was used to ensure that equal amounts of protein were loaded onto the gel. Blots are representative of three independent experiments. *C*, Day 4 MESC2.10 cells were transduced with LV-GFP or (*D*) LV-A53TSYN for 48 h and immunoprocessed with a mouse antibody against human α-synuclein (*red*). Confocal images were taken with a 40× objective. *E*, Day 4 MESC2.10 cultures were transduced with LV-GFP or LV-A53TSYN for 48 h, and basal [^{3}H]DA uptake and release were measured. Cells were loaded with [^{3}H]DA for 20 min at 37 °C and rinsed extensively with HBSS/Ca^{2+}. [^{3}H]DA uptake was then measured by lysing the cells immediately with 0.1 N perchloric acid/glutathione by freeze/thawing. Basal [^{3}H]DA release was measured by incubating cells in HBSS/Ca^{2+} for 20 min at 37 °C and measuring [^{3}H]DA in the supernatant. *F*, extracellular [^{3}H]DA release was induced by a 20-min exposure to 60 mM KCl or 50 μM AMPH. The supernatant was collected, and [^{3}H]DA was measured. All experiments were conducted in quadruplicate. Values are expressed as a percentage of LV-GFP control cells. The *graphs* represent the mean ± S.E. of three independent experiments. *, $p < 0.01$ compared with LV-GFP control (paired *t* test). #, $p < 0.01$ compared with LV-A53TSYN control (paired *t* test).

lentiviral vector. However, because we chose to examine a very large number of physiological parameters and MESC2.10 already express high levels of WT α-synuclein, we focused our efforts on studying the effects of A53TSYN expression. However, to control for the possible toxic effects of the lentiviral vector and of protein overexpression, we exposed control cultures to a lentivirus containing GFP, a reporter protein that is unlikely to have an effect on DA homeostasis. Transduction with LV-GFP led to a slight decrease in cell viability after 48 h (percentage of Calcein-AM-positive cells was 81.5 ± 8.4% compared with 100.0 ± 2.6% in untransduced cultures). Cell survival was not significantly different in cell cultures expressing A53TSYN (74.3 ± 8.1% cell viability).

Unlike most cell lines and primary culture cells currently used to study the pathogenicity of mutant α-synuclein (*e.g.* PC12, N27, B103, BE (2)M17, and HEK293 cell lines, and rat primary mesencephalic cells), differentiated MESC2.10 cells express high levels of WT human α-synuclein (Figs. 2*A*, 5*B*, *C*). Therefore we chose not to overexpress WT human α-synuclein in control cultures, even though the possibility that overexpression of the WT protein itself leads to pathogenicity cannot be excluded. Indeed, a recent study showed that adenoviral-induced overexpression of WT human α-synuclein in primary human mesencephalic cultures increased the rate of apoptosis in transduced DA neurons by 2-fold (44). In the present study, we used an m.o.i. that resulted in levels of A53TSYN that were only twice as high as those of endogenous WT α-synuclein to minimize a potential competitive effect between both mutant and WT proteins (Fig. 5*B*). Indeed, this 2-fold overexpression suggests that the ratio of mutant to WT protein is 1:1, as would be expected to occur in autosomal dominant forms of PD wherein one copy of the gene is mutant.

To examine the expression pattern of α-synuclein in differentiated MESC2.10 cells, Day 4 cells infected with either LV-GFP or LV-A53TSYN for 48 h were immunoprocessed with an antibody recognizing human α-synuclein. As shown in Fig. 5*C*, endogenous expression of WT α-synuclein was localized primarily to cell bodies in a pattern resembling that of early postnatal substantia nigra pars compacta neurons from rodents and humans (14, 45). Weak staining was also detected in the nucleus and cellular processes (Fig. 5*C*). MESC2.10 cells transduced with A53TSYN showed a dramatic increase in the neuritic expression of α-synuclein, although intense staining was also detected in the cell body (Fig. 5*D*). No gross abnormalities in cell shape, size, or neurite length were detected although a "thickening" of processes was consistently seen (Fig. 5*D*). Expression of α-synuclein in LV-A53TSYN-transduced cultures was confirmed by Western blot analysis, which showed a 2-fold increase in α-synuclein compared with the level of α-synuclein in LV-GFP-transduced cultures (Fig. 5*B*).

The effect of A53TSYN on the DA system of MESC2.10 cells was determined by studying three parameters: 1) changes in the expression of different DA markers by Western analysis, 2) [^{3}H]DA uptake and release, and 3) changes in intracellular DA immunofluorescence. In all cases, MESC2.10 cells were transduced with LV-GFP or LV-A53TSYN for 48 h. Expression of A53TSYN led to a 50% decrease in the levels of both DAT and VMAT2 when compared with cells transduced with LV-GFP (Fig. 5*B*). Interestingly, the expression of TH remained unchanged (Fig. 5*B*). Consistent with this decrease in DAT expression, cells expressing A53TSYN showed a 34.7 ± 4.4% decrease in [^{3}H]DA uptake (Fig. 5*E*). We observed an equivalent decline in the spontaneous release of [^{3}H]DA into the culture medium in cells expressing A53TSYN (28.4 ± 3.7%), which could have resulted from a reduced capacity of MESC2.10 cells to initially take up [^{3}H]DA (Fig. 5*E*).

Next, MESC2.10 cells overexpressing A53TSYN were examined for their ability to release DA in response to stimulation. Briefly, day 6 MESC2.10 cells transduced with either LV-GFP or LV-A53TSYN were loaded with [^{3}H]DA for 20 min and then exposed to either 60 mM KCl or 50 μM AMPH. Whereas MESC2.10 cells expressing GFP showed a 40.2 ± 4.8% increase in [^{3}H]DA release during to high potassium stimulation, MESC2.10 cells expressing A53TSYN did not release [^{3}H]DA in response to depolarization (Fig. 5*F*). However, they displayed an increased capacity to extrude [^{3}H]DA in response to AMPH compared with GFP-expressing cells (79.0 ± 13.4% *versus* 31.7 ± 2.6%, Fig. 5*F*). Because AMPH promotes transporter-mediated release of cytoplasmic DA, an increase in AMPH-induced release coupled to a decrease in exocytic release suggests that vesicular sequestration of [^{3}H]DA into synaptic vesicles is impaired in cells expressing A53TSYN. This could be

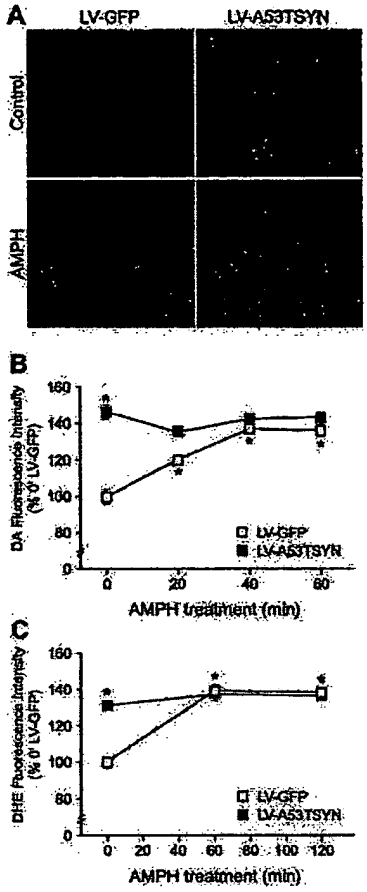

FIG. 6. **MESC2.10 cells expressing A53T mutant α-synuclein have enhanced basal levels of cytoplasmic DA and superoxide.** *A*, MESC2.10 cells were differentiated for 4 days and then transduced with either LV-GFP or LV-A53TSYN for 48 h. On day 6, cells were exposed to 50 μM AMPH for 20, 40, or 60 min, fixed, and immunoprocessed with a mouse antibody against DA. Confocal images, which were taken with a 60× objective, show DA-stained cultures exposed to AMPH for 60 min. *B*, the cytoplasmic DA fluorescence from ≥50 cells was measured per time point and is expressed as a percentage of 0 min for LV-GFP- and LV-A53TSYN-transduced cells. The *graphs* denote the mean ± S.E. of three independent experiments. *C*, same as in *A* except that transduced cells were exposed to 50 μM AMPH for 1 or 2 h. Cells were then incubated with 3 μM DHE, a superoxide-sensitive fluorophore, for 20 min at 37 °C and fixed with 4% paraformal dehyde for 30 min at 37 °C. Cytoplasmic DHE fluorescence was examined by confocal microscopy using a 60× objective. The fluorescence of ≥50 cells was measured per time point and is expressed as a percentage of 0 min for LV-GFP- and LV-A53TSYN-transduced cells. The *graphs* denote the mean ± S.E. *, $p < 0.01$ compared with LV-GFP 0 min (two-way ANOVA with post-hoc Dunnett's *t* test).

due to either a defect in transmitter uptake or to a depletion of synaptic vesicles available for storage.

To examine the effect of A53TSYN on intracellular DA distribution, we measured cytoplasmic DA immunofluorescence using an antibody against DA. DA immunofluorescence studies have been used by others to reliably detect changes in intracellular DA following exposure to DA-enhancing or -depleting compounds (*e.g.* Ref. 46). Day 6 MESC2.10 cells expressing GFP showed a granular pattern of DA immunofluorescence mainly localized to the cell body (Fig. 6*A*). On the other hand, MESC2.10 cells expressing A53TSYN showed increased levels of DA immunofluorescence both in the cell soma and in neurites, with bright granules of DA often seen along axons and in the extracellular space (Fig. 6*A*). Quantification of cytoplasmic DA immunofluorescence by confocal microscopy revealed a 46.8 ± 4.1% increase in DA fluorescence intensity in MESC2.10 cells expressing A53TSYN compared with cells expressing GFP (Fig. 6*B*). This increase in cytoplasmic DA fluorescence is consistent with the impaired ability of A53YSYN-expressing cells to release [^{3}H]DA in response to depolarization and again indicates a redistribution of vesicular DA to the cytosol (Fig. 5*F*).

Next, we examined the effect of A53TSYN on MESC2.10 cells exposed to a DA-redistributing dose of AMPH. GFP-expressing cells treated with 50 μM AMPH exhibited an increase in cytoplasmic DA immunofluorescence, which reached a plateau after 40 min of drug exposure (Fig. 6*B*, 37.8 ± 3.1% compared with untreated cells). The pattern of fluorescence resembled that of cells expressing A53TSYN. Interestingly, cells expressing the mutant protein did not display an increase in somal DA immunofluorescence in response to AMPH. In fact, fluorescence intensity decreased slightly in response to the psychostimulant (Fig. 6*B*), consistent with the greater ability of these cells to extrude DA through the DAT (Fig. 5*F*). MESC2.10 cells expressing A53TSYN did not exhibit an increase in DA immunofluorescence even after prolonged exposure to AMPH (144.3 ± 3.3% *versus* 146.8 ± 4.1% in untreated cells after 2 h).

Due to its unstable catechol ring, cytoplasmic DA can be rapidly oxidized to yield both ROS-like superoxide anion as well as reactive DA metabolites like DA-quinone (18–20). Thus, to determine whether increased cytoplasmic DA leads to enhanced intracellular production of ROS in MESC2.10 cells expressing A53TSYN, we monitored both LV-GFP- and LV-A53TSYN-transduced cells with the redox-sensitive fluorophore DHE. Because DHE is oxidized into fluorescent ethidium by superoxide anions, changes in DHE fluorescence can be used as an index of superoxide formation (47–49). Day 6 MESC2.10 cells expressing A53TSYN exhibited 31.0 ± 3.3% higher levels of basal DHE fluorescence than cells expressing GFP, suggesting that elevations in cytoplasmic DA result in increased production of superoxide radicals (Fig. 6*C*). Because the emission spectrum of DHE is >590 nm and GFP emits at 509 nm, GFP fluorescence did not overlap with our DHE measurements (this was confirmed by looking at GFP-expressing cells through a DHE filter).

Given that AMPH-induced distribution of vesicular DA into the cytoplasm can promote free radical formation (28, 50), coupled to the observation that MESC2.10 cells appear to be under a heightened state of oxidative stress, we questioned whether AMPH-induced superoxide formation was enhanced by expression of A53TSYN. Whereas MESC2.10 cells expressing GFP exhibited a 39.1 ± 3.6% increase in DHE fluorescence in response to a 60-min exposure to 50 μM AMPH, no changes were observed in response to A53TSYN expression, even after 2 h of drug exposure (Fig. 6*C*). Although quite surprising, these results suggest that MESC2.10 cells expressing A53TSYN are better able to deal with an AMPH challenge than cells expressing the WT protein. To test the possibility that A53TSYN-expressing cells scavenge DA-derived superoxide radicals more effectively than GFP-expressing cells when treated with AMPH, we examined the expression of cytoplasmic superoxide dismutase (Cu,Zn-SOD) in both types of cultures by Western immunoblotting. Protein levels of Cu,Zn-SOD in MESC2.10 cells expressing A53TSYN did not differ from those of GFP-expressing cells (Fig. 5*B*).

Expression of A53TSYN Confers Resistance to AMPH—Because MESC2.10 cells expressing A53TSYN exhibited higher basal levels of cytoplasmic DA and increased DHE fluorescence compared with cells expressing GFP, we questioned if they would be more sensitive to AMPH-induced toxicity. Thus, Day 3 MESC2.10 cells were exposed to LV-GFP or LV-A53TSYN for 24 h and then treated with 10–100 μM AMPH for 2 days in the continuing presence of virus. Because ≥80% of cells in Day 6

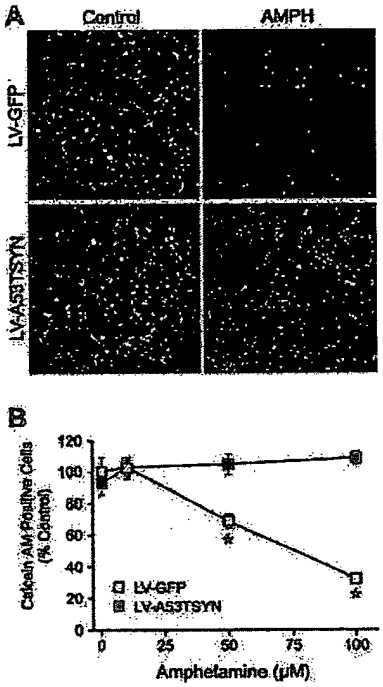

FIG. 7. **MESC2.10 cells expressing A53T mutant α-synuclein are resistant to AMPH toxicity.** *A*, Day 3 MESC2.10 cells were exposed to LV-GFP or LV-A53TSYN for 24 h and then treated with 10–100 μM AMPH for 2 days in the presence of virus. The *right panels* show cells exposed to 100 μM AMPH. On day 6, cell viability was assayed by incubating cells with 5 μM Calcein-AM for 20 min at 37 °C. Cells were visualized with a 10× objective. *B*, the total number of Calcein-AM-positive cells from five consecutive fields was counted per well. All data are expressed as a percentage of LV-GFP control. The *graphs* denote the mean ± S.E. of three independent experiments. *, $p < 0.01$ compared with LV-GFP control (two-way ANOVA with post-hoc Dunnett's *t* test).

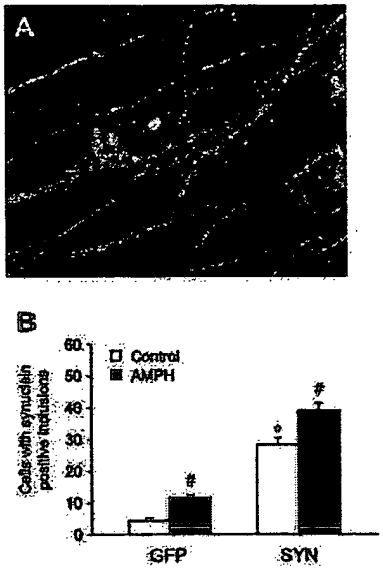

FIG. 8. **A53T mutant α-synuclein leads to inclusion formation.** *A*, MESC2.10 cells were differentiated for 4 days and then transduced with either LV-GFP or LV-A53TSYN for 48 h. On day 6, cells were exposed to 50 μM AMPH for 2 h, fixed, and immunoprocessed with a mouse antibody recognizing human α-synuclein. Shown is a confocal image taken with a 100× objective depicting both cytoplasmic and nuclear aggregates. *B*, the number of α-synuclein-positive inclusions from five consecutive fields was counted using a 60× objective. All data are expressed as a percentage of LV-GFP control. The *graphs* denote the mean ± S.E. of three independent experiments. *, $p < 0.01$ compared with LV-GFP control (one-way ANOVA with post-hoc Dunnett's *t* test). #, $p < 0.01$ compared with LV-A53TSYN control (one-way ANOVA with post-hoc Dunnett's *t* test).

cultures expressed TH, we assessed the viability using Calcein-AM, a membrane-permeable dye that is hydrolyzed in healthy cells by endogenous esterases into a membrane-impermeable fluorescent product. Transduction with LV-A53TSYN for 72 h did not lead to a significant decrease in cell viability compared with cells transduced with LV-GFP (Fig. 7*B*). Exposure of GFP-expressing cells to AMPH led to dose-dependent cell death, resulting in a 68.4 ± 2.9% loss of viability in cultures treated with 100 μM AMPH (Fig. 7, *A* and *B*). Surprisingly, MESC2.10 cells expressing A53TSYN were completely resistant to AMPH-induced toxicity (Fig. 7, *A* and *B*). Cell viability in LV-A53TSYN-transduced cultures treated with 100 μM AMPH was 91.5 ± 4.3% compared with 92.8 ± 8.1% in untreated cultures (Fig. 7*B*).

Lastly, because the appearance of protein aggregates has been inversely correlated to cell death both in PD and in Huntington's disease (51, 52), we looked for the presence of α-synuclein-positive inclusions in MESC2.10 cells expressing A53TSYN. We observed a dramatic increase in the number of MESC2.10 cells containing both cytoplasmic and intranuclear inclusions, the proportion of which was equal, in cultures transduced with LV-A53TSYN compared with cultures transduced with LV-GFP (39.1 ± 2.3% *versus* 4.3 ± 0.7%; Fig. 8, *A* and *B*). Moreover, a 2-h exposure to AMPH significantly increased the number of cells displaying α-synuclein-positive inclusions in LV-GFP and LV-A53TSYN-transduced cultures to a similar degree (Fig. 8*B*). The formation of intracellular aggregates in A53TSYN-expressing cells could contribute to their resistance to AMPH-induced toxicity.

DISCUSSION

Linkage of *α-synuclein* mutations to rare, autosomal dominant forms of PD (5, 6) has spurred a great interest in the mechanisms by which mutant α-synuclein leads to PD. Unfortunately, transgenic mice overexpressing WT or mutant α-synuclein do not reproduce the pathology of PD, and *in vitro* studies examining the effects of WT and mutant α-synuclein overexpression have yielded conflicting results. These discrepancies could be explained, in part, by the presence of a threonine instead of an alanine at position 53 in WT rodent α-synuclein or by fundamental differences between the *in vitro* systems studied and human nigral dopaminergic neurons. Therefore, we chose to study the effect of mutant α-synuclein in a new human mesencephalic cell line, MESC2.10. In this relevant model system, we tested the hypothesis that mutant α-synuclein leads to oxidative stress in nigral neurons by altering intracellular DA homeostasis.

Even though mice overexpressing WT human α-synuclein under the direction of the PDGF-β promoter exhibit a loss of striatal TH-immunoreactive terminals, they do not display reduced numbers of nigral TH-immunoreactive neurons nor fibrillar α-synuclein-positive inclusions like those found in PD (47, 48). Moreover, transgenic mice expressing WT and mutant α-synuclein under the direction of the mouse prion promoter show no pathology in the substantia nigra despite abundant expression of the transgene in this region (16, 17). Targeted overexpression of either A53T or A30P mutant human α-synuclein in nigral neurons does not lead to inclusion formation, DA depletion, cell loss (55), or increased sensitivity to MPTP toxicity (56) in aged mice. *In vitro*, overexpression of WT human α-synuclein in cultured cells has yielded contradicting results. Depending on the cell type studied, WT human α-synuclein increases (44, 57), decreases (58), or does not affect

(59) naturally occurring apoptosis. In most of these studies, rodent or peripheral human cell lines that do not express human α-synuclein were used. Thus, to study the effect of its mutant form, WT α-synuclein had to be introduced as a control. The incongruity of these findings could therefore be attributed to the differing cellular backgrounds in which WT human α-synuclein was overexpressed. In our case, A53T mutant human α-synuclein was introduced into a human mesencephalic cell line (Fig. 1) that exhibits properties of mature dopaminergic neurons (Fig. 2), providing an excellent model system for studying the pathogenicity of this protein. Because our cells already expressed human α-synuclein (Fig. 5, *B* and *C*), we chose not to introduce the WT protein. However, we cannot exclude the possibility that overexpression of the WT protein would have resulted in DA-related toxicity, as suggested by a recent study (44).

In most cases, overexpression of A53T mutant α-synuclein increases sensitivity to toxin-induced cell death. For instance, it exacerbates susceptibility of HEK293 and SH-SY5Y cells to DA toxicity (60, 61), of primary DA neurons and N27 cells to 6-OHDA-induced apoptosis (59), and of SH-SY5Y cells to MPP^+-mediated cell death (62). In contrast to these studies, MESC2.10 cells overexpressing A53TSYN were resistant to AMPH-induced toxicity (Fig. 7), a form of cell death thought to result from intracellular, DA-dependent oxidative stress (28, 50). Although the effect of WT or mutant α-synuclein on AMPH-induced toxicity has not been tested by other groups, recent studies suggest that cells overexpressing mutant forms of this protein are more sensitive to oxidative damage following addition of DA (60, 61) and 6-OHDA (59). The apparent discrepancy may be explained by high concentrations of applied DA and 6-OHDA acting as exogenous oxidant stressors not requiring the participation of endogenous transmitter stores or by differences in the cell types studied. In other words, application of exogenous dopamine may lead to both extracellular and intracellular formation of ROS, whereas AMPH primarily leads to the latter. Furthermore, we tested AMPH-induced toxicity in human mesencephalic neurons, while other groups measured DA- and 6-OHDA-mediated toxicity in either non-dopaminergic cells (*e.g.* HEK293) (54) or rat mesencephalic cells (53). Studies by Zhou and colleagues (44, 59) demonstrate that the pathogenicity of A53TSYN is not only dependent on the cell type used but on the species in which the mutant protein is expressed. For instance, although adenovirus-mediated overexpression of A53T mutant human α-synuclein in rat primary mesencephalic cultures led to a ≥60% decrease in TH cell viability compared with cultures transduced with GFP (59), human primary mesencephalic cultures transduced with the same vector displayed only a ≤20% loss of TH-positive cells (44).

Due to its unstable catechol ring, DA is readily oxidized in the cytoplasm into hydrogen peroxide, superoxide, and various DA metabolites (18). Intracellular accumulation of these toxic species can lead to detrimental cellular effects, including increased cytoplasmic calcium and protein and lipid damage (63). MESC2.10 cells overexpressing A53TSYN displayed 31% higher levels of superoxide compared with cells expressing GFP (Fig. 6*C*). Using the redox-sensitive dye dichlorofluorescein, Junn and Mouradian (61) also reported an increase in ROS production in SY-SH5Y cells overexpressing the mutant α-synuclein. One potential source of superoxide is unstored DA. Indeed, AMPH-induced elevations in somal DA immunofluorescence preceded increases in DHE fluorescence suggesting that superoxide radicals arose from the oxidation of cytoplasmic DA (Fig. 6, *B* and *C*). Furthermore, MESC2.10 cells expressing A53TSYN displayed a significant increase in basal DA immunofluorescence in the cell soma (Fig. 6, *A* and *B*) and an increase in AMPH-stimulated DA release (Fig. 5*F*), which suggests that cells expressing the mutant protein are unable to properly store DA into synaptic vesicles.

A gradual increase in oxidative stress may have triggered adaptive mechanisms in MESC2.10 cells that made them resistant to AMPH-induced toxicity. Indeed, repeated administration of AMPH to rats leads to an adaptive up-regulation of various antioxidant enzymes (64). To test whether exposure to mild oxidative stress during the hours following transduction with LV-A53TSYN led to a compensatory increase in antioxidant capacity, expression of the cytoplasmic superoxide scavenger Cu,Zn-SOD was assessed in MESC2.10 cells transduced with LV-A53TSYN for 48 h. No changes in Cu,Zn-SOD expression were observed in comparison to cells expressing GFP (Fig. 5*B*). This does not exclude the possibility that other antioxidant enzymes may have been up-regulated. In addition, resistance to oxidative stressors could be mediated by other factors, including the redox-sensitive transcription factor NF-kB (65). Interestingly, a similar resistance phenomenon has been observed during ischemic pre-conditioning, a condition whereby neurons exposed to sublethal cerebral ischemia become resistant to a subsequent lethal ischemic insult (66). In this case, enhanced mitochondrial calcium sequestration and/or calcium efflux is thought to be an important compensatory mechanism (67).

One possibility is that A53T mutant α-synuclein leads to an abnormal accumulation of cytoplasmic DA by reducing the number of vesicles in which neurotransmitter can be stored. Indeed, MESC2.10 cells expressing A53TSYN displayed a decrease in the levels of VMAT2, a marker of monoaminergic vesicles (Fig. 5*B*) and a reduction in depolarization-induced exocytotic DA release (Fig. 5*F*). The latter defect has also been observed in PC12 cells overexpressing the mutant protein (68). Even though α-synuclein binds to vesicles both *in vivo* (69) and *in vitro* (70), the A53T mutation does not affect its ability to bind to these structures (71, 72). Interestingly, α-synuclein is a potent inhibitor of phospholipase D isoform 2 (PLD2) (73), an enzyme that catalyzes the hydrolysis of phosphatidylcholine into choline and phosphatidic acid (74). *In vivo*, α-synuclein could modulate synaptic vesicle recycling via its regulation of PLD2 (75). This enzyme has been implicated in vesicle formation near the plasma membrane, given the ability of its product, phosphatidic acid, to recruit adaptor proteins and clathrin to membrane sites of vesicle budding (76). The possible role of α-synuclein in synaptic vesicle recycling is supported by a seminal study showing that suppression of WT α-synuclein by antisense oligonucleotides decreases the number of synaptic vesicles in the reserve pool (77).

Another mechanism whereby mutant α-synuclein could increase cytoplasmic DA might be through permeabilization of synaptic vesicles. Protofibrils of WT α-synuclein have been shown to bind and permeabilize phospholipid vesicles in a pore-like fashion, an effect that is enhanced by the A30P and A53T mutations (78, 79). In contrast, fibrillar α-synuclein oligomers are believed to be less cytotoxic (78), consistent with recent claims that fibrillar protein aggregates are harmless to cells (80). In addition, α-synuclein protofibrils appear to be stabilized by DA (81); increases in cytoplasmic DA could therefore promote further vesicle permeabilization. Alternatively, DA-induced oxidative damage of WT α-synuclein could also alter its ability to regulate PLD2 and worsen a mutant α-synuclein-mediated impairment in vesicle recycling.

A high proportion of MESC2.10 cells expressing A53TSYN exhibited aggregates immunoreactive for α-synuclein (Fig. 8). AMPH treatment also caused an increase in α-synuclein-positive inclusions (Fig. 8). Stressors such as ferric iron (82) or nitrating agents (83) promote α-synuclein aggregation, and the

increase in α-synuclein-immunoreactive aggregates observed in both cells overexpressing A53TSYN and GFP exposed to AMPH could result from cytoplasmic DA-derived ROS. Interestingly, not only is α-synuclein a major component of Lewy bodies (84), but mutant α-synuclein is more prone to self-aggregation than the WT protein (85, 86). Because surviving nigral neurons in PD-containing Lewy bodies appear to display less apoptotic features than neighboring Lewy body-free neurons (51), it has been suggested that these cytoplasmic aggregates might actually protect DA neurons from cell death. A similar phenomenon has been observed in the striata of early grade Huntington's disease patients, where a much higher proportion of spared interneurons than dying projection neurons display perikaryal huntingtin aggregates (52). Similarly, in a cell culture model of Huntington's disease, blockade of inclusion formation leads to decreased cell survival (87), and in a transgenic mouse model of the disease the number of striatal inclusions correlated with resistance to excitotoxin-induced striatal damage (88). Therefore, protein aggregates may indicate the activation of mechanisms that, at least temporarily, protect against cell death in slow neurodegenerative diseases.

In conclusion, the present study suggests that α-synuclein may play an important role in DA homeostasis by regulating neurotransmitter sequestration into synaptic vesicles. Our results demonstrate that expression of A53T mutant α-synuclein in a human mesencephalic cell line leads to an AMPH-like redistribution of DA from vesicular stores to the cytoplasm, which is accompanied by an increase in ROS. Additional studies are needed to assess whether overexpression of the WT protein can also disrupt DA homeostasis and to clarify whether mutant α-synuclein impairs DA storage by curtailing the formation of new synaptic vesicles or by interfering with neurotransmitter uptake. Moreover, it would be interesting to examine whether the protective mechanism observed in this study is gradually overrun so DA neurons expressing A53T mutant α-synuclein eventually succumb to disrupted DA metabolism, as may be the case in PD.

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Addendum—A recent report by Xu *et al.* (89) shows that depletion of DA by a TH inhibitor blocked apoptosis induced by overexpression of WT and mutant α-synuclein in primary human mesencephalic cultures. This study supports the role of dopamine in the pathogenicity of mutant α-synuclein, although overexpression of the WT protein had a similar effect.

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